IMMUNOPOTENTIATING EFFECT OF AMPHOTERICIN B ON RESISTANCE TO EXPERIMENTAL CANDIDA INFECTION IN MICE

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Summary. - Amphotericin B (AmB) is a polypene antifungal antibiotic that has immunomodulatory properties. This led us to think that at least part of antifungal activity may be attributed to its immunomodulatory properties. In an approach to this problem we investigated the impact of AmB treatment on host resistance to C. albicans as measured under both in vivo and in vitro conditions. Mice receiving a single intraperitoneal injection of AmB showed increased resistance to subsequent challenge with either C. albicans or S. aureus. The protective effect of AmB was conditioned by dose, time of drug administration and size of yeast, and was reversed by cyclophosphamide. In vivo augmentation of resistance to experimental C. albicans infection coincided with the appearance in the spleen of a highly candidacidal cell population with properties of activated macrophages. Reactive in vitro against 51Cr labelled yeast cells. Furthermore, normal macrophages from intact mice can be activated in vitro by AmB to become cytotoxic against C. albicans cells. The results of this study suggest that AmB is capable of activating cells in the host with increased candidacidal activity and this effect may contribute significantly to its therapeutic activity. This observation can give us a better understanding of the mechanisms underlying the therapeutic efficacy of AmB as an antifungal agent.

Introduction

Amphotericin B (AmB) is a polypene macrolide antibiotic produced by several species of Streptomyces [1] used principally in the treatment of systemic fungal infections [2]. This antibiotic causes fungal cell death by binding to sterols in the cell membrane [3] which first leads to the leakage of potassium [4] followed by cell contents [5]. These effects are blocked in the media by ergosterol, which binds to AmB and prevents it from adhering to the fungal cell membrane [6]. It has been suggested that the formation of AmB-cholesterol complexes can result in pores through the membrane lipid bilayer [7] which causes death of the cell.

AmB has a narrow therapeutic range. In high concentrations it may cause hypokalemia and renal acidosis, as an effect on the permeability of cell membranes [8], but at therapeutic concentrations clinical side effects normally are of limited significance. Many studies have demonstrated that AmB produces dramatic immunomodulating effects on the immune system. In mice, its use in correlation with
certain antitumor agents results in cures of AKR leukemia under conditions in which none was produced by either agent alone [9].

Multiple studies demonstrated that AmB produces immunomodulatory effects. Indeed, AmB alters T-cell activity, augmenting graft versus host reactions [10], enhances contact sensitivity [11], increases in vitro proliferative responses [12], and induces generation of suppressor T lymphocytes and monocytes [13]. Furthermore, AmB increases secondary in vitro antibody formation [14] and acts as a polyclonal B cell mitogen [15].

AmB also increases the resistance of mice to several microbial infections. AmB-treated mice resist infection with Lysteria monocytogenes [16]; such protection is not due to a direct toxic effect of the drug on the organism but rather to enhanced phagocytic and microbicidal activity of host macrophages [17]. AmB, furthermore, enhances resistance to infection with the helminth Schistosoma mansoni by activation of murine macrophages which results in greater killing of this multicellular parasite [18].

In addition to modifying anti infectious activity of macrophages, AmB also enhanced tumoricidal activity in macrophages from mice chronically infected with bacillus Calmette-Guerin [19]. These results presented the question whether at least part of the antifungal activity of AmB may be attributed to its immunoactive properties. In an approach to this problem, and following a line of experimental research in our laboratory on modulation of resistance to Candida albicans infection in mice by drugs [20], we investigated the effect of AmB treatment on host resistance to the yeast as measured under both in vivo and in vitro conditions.

Effect of AmB administration on in vivo resistance against microbial challenge

In a first series of experiments we assessed the kinetic pattern of AmB-induced modifications in the susceptibility to C. albicans infections by studying the effect of drug treatment induced at different times before systemic challenge with the yeast. The results indicated that AmB treatment (10 mg/kg) 2 to 8 days before microbial challenge increased resistance both to C. albicans and S. aureus infection. However, the beneficial activity exerted by AmB pretreatment was completely abrogated by exposure of mice to Cyclophosphamide (Cy) (150 mg/kg) 3 days after AmB treatment (AmB day –8, with respect to microbial challenge, day 0). The antagonistic effect of Cy was strictly dose dependent (data not shown).

The modifications in the susceptibility to C. albicans infection were related to the in vivo killing of the injected yeast. Indeed, animals given AmB at differences times before C. albicans challenge and assayed for survival of the yeasts in the kidney and spleen showed that both organs followed different patterns of C. albicans growth, depending on the treatment schedule. When AmB was given 3h after challenge, there was an immediate inhibition of fungal

Table 1. Effect of AmB administration to CD2F1 mice on various parameters of resistance to C. albicans and antagonistic effects of Cy

<table>
<thead>
<tr>
<th>AmB (mg/kg)</th>
<th>% 51Cr specific release (c)</th>
<th>Challenge i.v. with 1x10⁷ C. albicans cells (e)</th>
<th>CFU recovered from kidney of mice infected with 1x10⁸ C. albicans cells (f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.6 7.8 4.1</td>
<td>8.5 10/10</td>
<td>1052 Dead</td>
</tr>
<tr>
<td>10</td>
<td>21.6* 14.5* 8.4*</td>
<td>&gt; 60* 0/10</td>
<td>35* 1.4</td>
</tr>
<tr>
<td>10</td>
<td>150 4.1 2.3 1.6</td>
<td>3 10/10</td>
<td>2680 Dead</td>
</tr>
<tr>
<td>10</td>
<td>150 6.3 3.4 2.5</td>
<td>4 10/10</td>
<td>1658 2459</td>
</tr>
</tbody>
</table>

(a) AmB was given a single i.p. injection 8 days before C. albicans challenge or in vitro assay (day 0).
(b) Cy was given as a single i.p. injection 3 days before C. albicans challenge or in vitro assay.
(c) CD2F1 mice were donors of effector spleen cells to be reacted against radiolabelled C. albicans cells in a 4h 51Cr release assay.
(d) Effector to target cell ratios.
(e) Mice were injected i.v. with 1x10⁷ C. albicans cells.
(f) MST, median survival time.
(g) D/T, dead mice at 60 days over total number of animals tested.
(h) Mice were injected intravenously with 1x10⁸ C. albicans cells; groups of mice were sacrificed 2 or 4 days after infection and their organs were processed for assessment of multiplication of the yeast. Results are expressed as mean ± standard error of the mean, and so the standard errors have been omitted.

* p < 0.01 (treated versus untreated control).
proliferation. When performed 8 days earlier AmB treatment resulted in initial growth (20 to 40h) of the microorganism which was later cleared (Table 1).

Further studies by us evidenced the existence of AmB-mediated effects on host responsiveness. First, despite the fact that AmB has a prolonged elimination half-life with rather complex pharmacokinetics, in our experimental model, the concentration of the drug in plasma after a single dose of 10 mg/kg fell below the lower limit of detection by bioassay within 72 h. Thus, direct interaction of the drug with C. albicans can be ruled out when we consider the longer-term effects of AmB. Second, increased resistance was also observed against an AmB resistant organism, such as S. aureus. Third, the longer term beneficial effects of AmB treatment against C. albicans challenge were reversed by subsequent exposure of mice to Cy, which is known to suppress the natural defense mechanisms of the host.

**In vitro analysis of effector cells with candidacidal activity induced by AmB treatment**

By using two reliable tests for quantitating the ability of different murine effector cells to in vitro killing of C. albicans cells, as radiolabel release from 51Cr-labelled yeast cells and inhibition of CFU formation, we were able to demonstrate that AmB treatment led to considerable activation of adherent cells present in the spleen and peritoneal exudate, whereas it had no significant effect on polymorphonuclear neutrophils and bone marrow cells. Anti-Candida cytotoxic effectors appear in the spleen and peritoneal cavity after treatment with AmB and have the morphological and functional properties of macrophages. It is important however to underline that the effects of AmB administration both on resistance and candidacidal activity were dependent on the treatment schedule. A single dose of 10 mg/kg administered 8 days before Candida challenge was as effective as daily (days -12 to -8) exposures to AmB at a dose of 2 mg/kg. The same fractionated dose, on the other hand, was largely ineffective when administered every other day from day -16 to -8. Similar results were obtained with AmB at a dose of 50 mg/kg.

In further experiments it seemed of interest to evaluate the effect of AmB treatment on a second challenge of mice with Candida, long after (15 days) the first challenge, at a time when AmB could no longer express immunoprotective activity, (i.e. 16-22 days after drug treatment). The results indicated that the animals that had resisted the first challenge could survive the second one, regardless of the time interval between drug exposure and the second Candida injection. This seems to suggest that the protection to reinfection was not a direct effect of drug treatment, but rather resulted from development of immunity following the first yeast injection (Table 2).

**Table 2. - Effect of AmB administration on resistance of CD2F1 mice to repeated C. albicans infections**

<table>
<thead>
<tr>
<th>AmB administration</th>
<th>First challenge with C. albicans (day 0)</th>
<th>Second challenge i.v. with C. albicans (day + 15) (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>Route</td>
<td>MST (d)</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>----------</td>
</tr>
<tr>
<td>Untreated</td>
<td>1x10⁶</td>
<td>i.v.</td>
</tr>
<tr>
<td>Untreated</td>
<td>2x10⁶</td>
<td>i.v.</td>
</tr>
<tr>
<td>Untreated</td>
<td>8x10⁶</td>
<td>i.v.</td>
</tr>
<tr>
<td>Untreated</td>
<td>2.5x10⁷</td>
<td>i.p.</td>
</tr>
</tbody>
</table>

(a) AmB (10 mg/kg) was given as a single i.p. injection 2 or 8 days before the first C. albicans challenge (day 0).

(b) Mice surviving 15 days after the first C. albicans challenge were injected i.v. with 1x10⁶ C. albicans cells.

(c) MST, median survival time.

(d) D/T, dead mice at 60 days over total number of animals tested.

* p < 0.01 (AmB-treated versus untreated controls).

**In vitro activation of macrophages by AmB**

Plastic adherent spleen cells after in vitro incubation with AmB showed strong anti-Candida activity. This phenomenon was dose and time dependent. There is a threshold value (10 µg/ml) below which no significant activation can be obtained and maximal candidacidal activity was present after 2h of incubation with AmB. Macrophage activation which was evident in both the 51Cr-release assay and CFU inhibition assay gradually declined when the cells, activated either in vitro or in vivo were kept under in vitro conditions for varying lengths of time before testing. It was found that the phagocytic efficiency of AmB-activated macrophages was considerably enhanced with respect to untreated controls both in terms of kinetic parameters of phagocytosis as well as total number of Candida cells ingested. Moreover, on incubation of AmB-activated macrophages with live C. albicans cells, marked inhibition of hyphae formation was obtained (Table 3). In vivo or in vitro AmB activated macrophages after adoptive transfer in Cy-depressed mice are able to partially recover resistance to systemic infection with C. albicans.

In conclusion, our results strongly indicate that AmB is capable of increasing in vivo or in vitro candidacidal activity of macrophages. This effect in the host may contribute significantly to its therapeutic efficacy and it seems particularly important since the clinical efficacy of AmB can be crucial in helping to overcome low immune responses in conditions in which immune deficient hosts have developed chronic systemic infection.
Table 3. Effect of time and concentration of AmB incubated in vitro with normal adherent spleen cells on their phagocytic and candidacidal activity

<table>
<thead>
<tr>
<th>In vitro incubation with AmB (a)</th>
<th>% $^{51}$Cr specific release (b)</th>
<th>% CFU inhibition (c)</th>
<th>% of cells which ingested at least one C. albicans cell (d)</th>
<th>Number of C. albicans cells ingested per 100 adherent cells (e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (µg/ml)</td>
<td>Time (hours)</td>
<td>10:1</td>
<td>5:1</td>
<td>2.5:1</td>
</tr>
<tr>
<td>1 1h</td>
<td>10.1</td>
<td>7.0</td>
<td>4.3</td>
<td>39.4</td>
</tr>
<tr>
<td>1 1h</td>
<td>14.1</td>
<td>9.7</td>
<td>6.4</td>
<td>54.6*</td>
</tr>
<tr>
<td>10 1h</td>
<td>24.6*</td>
<td>17.8*</td>
<td>12.6*</td>
<td>85.9*</td>
</tr>
<tr>
<td>1 4h</td>
<td>14.3</td>
<td>10.4</td>
<td>6.8</td>
<td>56.7</td>
</tr>
<tr>
<td>4 4h</td>
<td>17.3</td>
<td>11.4</td>
<td>8.6</td>
<td>70.9*</td>
</tr>
<tr>
<td>10 4h</td>
<td>28.5*</td>
<td>23.3*</td>
<td>18.9*</td>
<td>98.4*</td>
</tr>
</tbody>
</table>

(a) Plastic adherent cells from CD2F1 mice were cultured for 1 or 4 h with 1 or 10 µg/ml of AmB.
(b) Candidacidal activity was assayed against radiolabeled C. albicans cells in a 4 h $^{51}$Cr-release assay.
(c) Effector to target cell ratios.
(d) The assay was performed with an incubation of 4 h.
(e) Adherent spleen cells from normal or AmB treated mice were incubated in vitro for 4 h with inactivated C. albicans cells at a ratio of 1:5.
(f) Number of phagocytosed cells for 100 adherent cells after 4 h of in vitro incubation.

* p < 0.01 (AmB treated versus controls).

REFERENCES


TRANSFERRINS AND DEFENCE AGAINST INFECTION

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Summary. – This paper reviews the role of transferrin in immunity and defence against infection. The transferrins (serum transferrin, lactoferrin and ovotransferrin) are all glycoproteins capable of reversibly binding two ferric ions. The main function of serum transferrin is to supply iron to cells, and this event is important during the proliferative response of lymphocytes. Consequently, iron deficiency may impair immune responsiveness by decreasing the availability of transferrin-bound iron. The transferrins may also have a direct antimicrobial effect: because of their high affinity for iron, there is normally no «free» iron available to bacteria within the body. However, severe iron overload may result in complete saturation of serum transferrin and loss of antibacterial activity, and furthermore some pathogenic bacteria are able to overcome the effect of transferrin by producing competing low molecular weight chelators (siderophores). Some siderophores are used in chelation therapy or iron overload, and their potential for stimulating microbial growth should be borne in mind. Patients undergoing continuous ambulatory peritoneal dialysis (CAPD) may likewise be at risk because of dilution of transferrin by dialysis fluid in the peritoneal cavity. Lactoferrin in human milk may exert an antimicrobial effect in the neonatal gut and protect the breast fed infant against gastrointestinal infections.

Riassunto (Transferrine e difesa antinfettiva). – È passato in rassegna il ruolo della transferrina nell'immunità e nella difesa antinfettiva. Il sistema delle transferrine (transferrina serica, lactoferrina e ovotransferrina) è costituito da glicoproteine, che legano ferro, la cui principale funzione è di fornire ferro alle cellule. Questa funzione è importante durante la proliferazione linfocitaria e pertanto la deficienza di ferro può abbassare la risposta immunitaria. Le transferrine possono avere un effetto antimicrobico diretto: infatti, dati la loro alta affinità per il ferro, non c'è ferro libero in circolo disponibile per i batteri. Un sovraccarico di ferro può saturare la transferrina con conseguente perdita dell'attività batterica. Inoltre alcuni batteri patogeni competono con il ferro della transferrina tramite chelanti a basso peso molecolare, i siderofori. I pazienti sotto dialisi possono risalire a rischio infettivo data la continua diluzione della transferrina data dal liquido dialitico peritoneale. La lattoferrina può esercitare un effetto antibatterico nell'intestino del neonato e proteggere il lattante dalle infezioni gastrointestinali.

Introduction

Transferrin occupies a central role in iron metabolism. Its main function is to transport iron from sites of absorption and storage to the bone marrow, for incorporation into developing erythrocytes. This function of transferrin is carried out as a result of the protein’s unique property of being able to reversibly bind two ferric ions at specific sites. Although the total amount of iron bound to transferrin at any given time is only about 0.1 % of the total body iron, variation in this pool can have important consequences not only on erythrocyte production but also, as discussed in detail below, on susceptibility to infection.

A related protein, lactoferrin, is found in neutrophil secondary granules, and in milk and a variety of other secretions. The function of lactoferrin remains unclear: there is little evidence of an iron-transport role, but it may, like transferrin, contribute to antimicrobial defences. A third protein, ovotransferrin, is found in avian egg white and closely resembles its serum counterpart.

In this article the structure and function of the transferrins will be briefly reviewed, and this will be followed by a discussion of the way in which abnormalities in their levels, structure and degree of iron saturation may increase the risk of infection. For a more extensive review of transferrin structure and function see Brock [1].
Structure of the transferrins

The structures of transferrin and lactoferrin are very similar. Both are single-chain proteins of molecular weights about 78,000 and 85,000 respectively, folded into two globular domains [2, 3]. Both proteins are glycoproteins, each containing two carbohydrate chains. Each domain carries one of the iron-binding sites, and it is possible, by limited proteolysis, to cleave the proteins in such a way that isolated domains with intact iron-binding sites can be obtained [4-6]. One major difference between transferrin and lactoferrin is their isoelectric points; the pI of transferrin is in the range 5.4-5.9, depending on whether or not iron is bound [7], but that of lactoferrin is unusually high, values in the region of 8.4-9.0 having been reported [8]. This may account for the fact that lactoferrin readily forms non-covalent complexes with a variety of proteins and other substances [9], a property which may enable it to remain associated with mucosal surfaces.

Iron binding at the specific sites requires the presence of a bicarbonate anion, and no iron binding occurs if bicarbonate or CO₂ are rigorously excluded [10]. The affinity of transferrin for iron at physiological pH and pCO₂ is extremely high, being of the order of 10²⁰ M⁻¹ [11], so that iron, once bound, will not dissociate from the protein. However, the affinity falls rapidly as the pH is lowered, and at pH 5-6 iron will be removed even by relatively weak chelating agents such as citrate [12]. With lactoferrin, however, destabilization by citrate does not occur until the pH drops to about 2 [13]. This difference may allow lactoferrin to maintain its iron-binding properties in acidic environments such as the gastrointestinal tract.

The transferrins will bind a variety of transition metals and lanthanide ions at the iron-binding sites (summarized in ref. [11]), but all such complexes so far investigated are less stable than those with iron, and with the possible exception of zinc [14], there is little evidence to suggest that any are bound in vivo. Ferrous iron is bound, if at all, only with very low affinity [15].

Cellular uptake of transferrin-bound iron

The major function of transferrin is to supply iron to those cells which require the metal. Most transferrin-bound iron is destined for incorporation into haemoglobin in developing erythrocytes, but smaller amounts are also required by non-erythroid cells when undergoing proliferation. Given the extremely high affinity of transferrin for iron under physiological conditions, it is evident that some specialised mechanism must exist to allow cells to remove iron from transferrin. It is now generally agreed that this occurs by a rather unusual type of receptor-mediated endocytosis [16]. Iron-requiring cells express membrane receptors for transferrin, which preferentially bind iron-containing transferrin molecules. The iron-transferrin-receptor complex is then internalized in a vesicle in which the pH drops to 4.5-5, causing the iron to be released from transferrin. The vesicle then fuses with the cell membrane, and the iron-free (apo)transferrin is displaced from the receptor by a new iron-containing transferrin molecule. Thus both transferrin and its receptor are preserved during the iron-uptake process.

There is little evidence that lactoferrin fulfills any comparable iron-transport role. It has been reported that mucosal cells express lactoferrin receptors and acquire lactoferrin-bound iron \textit{in vitro} [17, 18], but \textit{in vivo} data does not provide any evidence of a positive role for lactoferrin in iron absorption [19].

Transferrin acquires iron \textit{in vivo} mainly from those macrophages of the liver and spleen which are involved in the catabolism of effete erythrocytes. Under normal conditions the release of iron to transferrin seems to be linked to erythropoietic needs [20], though how this is achieved is unclear, as the degree of iron saturation of transferrin itself does not appear to be the regulatory factor [21, 22].

Antimicrobial properties of the transferrins

Given the high affinity with which transferrins bind iron at physiological pH, it is evident that «free» iron, or iron bound to low affinity ligands is likely to be sequestered by these proteins as long as binding sites remain available. Most bacteria, and virtually all pathogens, have a significant iron requirement, and hence their growth in body tissues will be impeded by lack of readily-available iron. This antibacterial property of the transferrins was first demonstrated \textit{in vitro} by Schade and Caroline over 40 years ago [23], and has been amply confirmed in subsequent studies which have shown that many species of bacteria, when inoculated into serum, human milk or media containing purified transferrins, fail to grow [24]. Bacteriostasis can be overcome by adding sufficient iron to saturate the transferrin present.

In some cases, however, the bacteriostatic effect of transferrins is only temporary. Moreover, it is evident that pathogens capable of causing disease in man must possess some means of overcoming the non-availability of iron. It is now known that many bacteria can produce low molecular weight iron chelators of high affinity known as siderophores, which are capable of competing with transferrins for iron [2]. The iron-siderophore complexes are then taken up by bacteria via specific outer-membrane receptors. Many different siderophores have now been identified, and the structure and mode of action of those produced by bacteria liable to cause disease in the immunocompromised host is dealt with elsewhere in this volume. However, it will be evident
that in the initial phase of microbial infection, the outcome will depend on whether the invading organisms can multiply sufficiently rapidly to outstrip the action of phagocytic cells and other host microbicidal activities. Consequently any factor which tips the balance in favour of microbial proliferation will predispose to overt infection, and the ability of a microorganism to express iron acquisition mechanisms will increase its pathogenicity.

While transferrin helps to prevent microbial growth in plasma and tissues, lactoferrin may perform a similar role at mucosal surfaces, and participate in the antimicrobial activity of neutrophils.

Transferrins and the immune response

The immune response is a complicated sequence of events involving a variety of cells and soluble factors. However, a key event is the expansion of clones of lymphocytes to produce antibody-secreting plasma cells or various types of effector T-cells. Since transferrin-bound iron is necessary for cell proliferation, it might be expected to be important in lymphocyte proliferation, and this is indeed the case. It has been shown that if lymphocytes are cultured with mitogens in media without serum, proliferation only occurs if transferrin is added [26]. Other forms of iron cannot substitute for transferrin, and if the iron saturation of transferrin is very low, the rate of proliferation is decreased [26, 27]. Furthermore, it has been shown that supplementation of lymphocyte cultures with serum from iron-deficient mice does not permit optimal proliferation due to the reduced transferrin saturation [28]. On the other hand, excess iron may also reduce proliferation [26]. It has been shown that in activated T-lymphocytes expression of transferrin receptors is triggered by the binding of interleukin-2 (IL-2) to the Tac (IL-2) receptor [29], and this is then followed by binding of transferrin, uptake of iron, and proliferation (Fig. 1) [30]. In B-lymphocytes, however, there is evidence that some synthesis of immunoglobulin may occur even in the absence of transferrin binding and subsequent proliferation [31]. The requirement for transferrin-bound iron is probably related specifically to proliferation rather than to subsequent events such as release of lymphokines, as protein synthesis by activated lymphocytes is not influenced by the degree of iron-saturation of transferrin [28].

Changes in transferrins and increased risk of infection

Iron deficiency - Decreased transferrin saturation

Severe iron deficiency reduces the saturation of serum transferrin from around 30% to 10% or less. Since delivery of iron to proliferating lymphocytes may be impaired under these circumstances, one might expect evidence of depressed immune responses, and a number of clinical studies support this

![Diagram of T-lymphocyte activation showing the role of transferrin in proliferation.](image)
suggestion. Cell-mediated immune responses in particular are decreased in such patients [32-35], but there is less evidence of impaired antibody responses, which may reflect the fact that transferrin-bound iron is not essential for immunoglobulin synthesis.

Whether these impaired cell-mediated immune responses increase susceptibility to infection is less clear. Chronic mucocutaneous candidiasis seems to be associated with iron-deficiency [36, 37], and it is generally agreed that iron-deficient infants tend to be more susceptible to infection [38, 39]. However, there is little evidence that resistance to tuberculosis, in which cell-mediated immunity is classically involved, is impaired by iron deficiency. Hence defects in T-lymphocyte activity resulting from reduced transferrin saturation may not in themselves be sufficient to cause increased susceptibility to infection. Nevertheless, the likelihood of reduced immunocompetence should always be born in mind when dealing with iron-deficient patients.

Finally, it is unlikely that the decreased transferrin saturation associated with iron deficiency can enhance the antimicrobial activity of transferrin, as even under normal conditions transferrin remains only about 30% saturated with iron.

Iron overload - Increased transferrin saturation

There are several conditions that can result in transferrin having an abnormally high iron saturation [40]. In patients with severe iron overload resulting from primary haemochromatosis or transfusion therapy for thalassaemia transferrin may become completely saturated with iron. Liver damage exacerbates these conditions, and may in itself cause increased transferrin saturation. In patients whose plasma transferrin is saturated a pool of iron bound loosely and non-specifically to other serum proteins is found [41], and such iron will be more readily available to microbial iron-scavenging mechanisms than transferrin-bound iron. There is ample evidence that such sera have reduced bacteriostatic activity in vitro [42], and some evidence of an associated increase in septicaemic infections [43, 44]. In less severe conditions where transferrin saturation, though elevated, is < 100%, there is no additional pool of plasma iron [45], but increased incidence of septicaemic infections is still noted, especially if liver damage has occurred [42]. Thus while saturation of serum transferrin may not be the only cause of increased incidence of infection in iron overload, it undoubtedly does contribute to the increased risk.

In infants, there have been reports of an increased incidence of septicaemic infections following treatment of anaemia by parenteral administration of iron dextran [46, 47]. Persistence of iron dextran in the circulation may provide microorganisms with an alternative source of iron.

Chelation therapy

In certain clinical conditions of iron overload the level of excess iron may be reduced by treating the patients with the iron-chelating drug desferrioxamine. While removal of excess iron is obviously desirable, and may contribute to increased resistance to infection in the long-term, it must be remembered that desferrioxamine is itself a siderophore, and can be utilized by a number of microorganisms, including some pathogens [48]. Chelation therapy may therefore actually increase the risk of infection, as is demonstrated by the fact that infants with acute iron overload due to accidental ingestion of iron tablets became infected with Yersinia enterocolitica when treated with desferrioxamine [49]. Although the iron acquired by desferrioxamine is probably of intracellular origin rather than from transferrin itself, the presence of the iron-siderophore complex can aggravate the protective effect of transferrin.

Reduced transferrin levels

a) Transferrin and continuous ambulatory peritoneal dialysis (CAPD). - The normal levels of transferrin in serum and extravascular fluids are well above those necessary to provide good inhibition of growth of most pathogenic bacteria. Abnormally low levels of serum transferrin, and increased susceptibility to infection have been reported in congenital atransferrinaemia [50, 51], but this is an extremely rare condition. However, a much more frequent clinical situation in which very low transferrin levels are present in body fluids occurs in patients undergoing CAPD for kidney failure. These patients have a marked tendency to develop peritonitis, with Staphylococcus epidermidis being the most frequent causative organism. The patients receive infusions of large volumes of fluid into the peritoneal cavity, and we have found that in this fluid the transferrin level is only about 2% of that in serum or normal peritoneal fluid (Table 1). In vitro growth of S. epidermidis was significantly greater in dialysis fluid than in serum or normal peritoneal fluid. Moreover there was a significant inverse correlation between growth in CAPD fluid and transferrin concentration, but no such correlation was found for serum or normal peritoneal fluid. These results suggest that the transferrin antimicrobial system does not function optimally in the peritoneal cavity of patients undergoing CAPD, and this might be partly responsible for their susceptibility to peritonitis.

b) Decreased lactoferrin levels. - It has been reported that patients suffering from a congenital defect of lactoferrin production by neutrophils are susceptible to infection with organisms such as Staphylococcus aureus [52]. Again, however, this is a rare condition. A much more frequent situation
Table 1. - *Transferrin levels and growth of S. epidermidis in serum and peritoneal fluid*

<table>
<thead>
<tr>
<th></th>
<th>No. of samples</th>
<th>Transferrin (mg/dl)</th>
<th>Growth of S. epidermidis (**)</th>
<th>P (*)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>mean (range)</td>
<td>mean (range)</td>
<td></td>
</tr>
<tr>
<td>Normal serum</td>
<td>6</td>
<td>257 (210-230)</td>
<td>11.8 (5.0-15.3)</td>
<td>—</td>
</tr>
<tr>
<td>CAPD patients' serum</td>
<td>17</td>
<td>211 (105-420)</td>
<td>3.8 (0-34.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Normal peritoneal fluid</td>
<td>8</td>
<td>229 (200-305)</td>
<td>6.6 (0.7-16.0)</td>
<td>NS</td>
</tr>
<tr>
<td>CAPD dialysis fluid</td>
<td>44</td>
<td>4.7 (0.3-16.3)</td>
<td>23.6 (0.8-105)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

(*) Compared with normal serum.
(**) As percentage of uninhibited growth in tissue culture medium.

in which the absence of lactoferrin may predispose to infection is in the bottle-feeding of infants. There is sufficient lactoferrin in breast milk to inhibit the growth of enteropathogenic *Escherichia coli* in vitro, and animal studies suggest that the effect may be important in vivo [19]. The increased susceptibility of bottle-fed infants to gastrointestinal infections may therefore be due, at least in part, to the virtual absence of lactoferrin from formula feeds. Nevertheless, recent clinical studies have produced conflicting results: Corda et al. [53] found that feeding of ovotransferrin to infants resulted in a more rapid recovery from enteritis, but Moreau et al. [54] found that addition of bovine lactoferrin to formula did not alter the faecal coliform flora of healthy infants. Further clinical studies in this area are needed.

Abnormalities in transferrin iron-binding

There is only one report of a congenital defect in transferrin which affected the protein's iron-binding properties [55], and there was no evidence of an associated increase in infections. In patients with idiopathic haemochromatosis the serum transferrin appears to bind iron more slowly than normal [56], which might contribute to the susceptibility of these patients to infection (see above). However, the cause of the abnormality was not investigated. Finally, it has been suggested that the susceptibility of patients with diabetic ketoacidosis to mucormycosis may be due to the lowered serum pH, which reduces the affinity of transferrin for iron and thus makes the metal more readily available to the infecting fungi [57].

REFERENCES


ATTIVITÀ ANTIMICROBICA DELLE TRANSFERRINE

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Riassunto. — Le transferrine degli organismi superiori svolgono un’azione antimicrobica in virtù della loro capacità di chelare due atomi di ferro per molecola. A questa azione i microbì si oppongono mediante la sintesi di siderofori di natura catecolica o idrossamiche, la cui sintesi può essere sotto codificazione cromosomica o plasmidica. Fra i più tipici rappresentanti di microbì opportunisti sono state prese in considerazione Pseudomonas aeruginosa e Candida albicans. Nei confronti di questa seconda specie l’azione delle transferrine appare essere più complessa e non legata esclusivamente alla chelazione del ferro.

Summary (Antimicrobial activity of transferrins). — Transferrins of higher organisms have an antimicrobial action by virtue of their capacity of chelating two iron atoms per molecule. Microbes antagonize this action by synthesizing catecol or hydroxamic siderophores which can be coded by the chromosome or by plasmids. Among the most typical species of opportunistic microbes Pseudomonas aeruginosa and Candida albicans have been considered. Towards the latter the action of transferrins appears to be more complex and not connected exclusively to the iron chelation. This was demonstrated by using transferrins saturated with different metal ions, bound to Sepharose 4B or separated from the microbial culture by means of a dialysis membrane.

Fra i vari meccanismi di difesa degli organismi superiori nei confronti dei microbi parassiti, il sistema delle transferrine ha suscitato in questi ultimi anni un interesse crescente. È noto infatti come in natura tutti gli esseri viventi abbiano bisogno di un certo numero di elementi chimici essenziali, e tra questi il ferro ha una importanza fondamentale quale componente di vari enzimi ed in particolare dei sistemi di trasporto degli elettroni nelle catene respiratorie. Benché largamente diffuso nella biosfera, questo metallo sotto forma di ione ferro è praticamente insolubile in acqua e necessita quindi di una solubilizzazione da parte degli esseri viventi prima di essere utilizzato. Questa solubilizzazione, con conseguente trasporto, avviene negli organismi superiori mediante l’azione di glicoproteine di peso molecolare di circa 80,000, in grado di chelare due atomi di ferro per molecola, e rappresentate nei mammiferi dalla sierotransferrina e dalla lactotransferrina.

La capacità di legare il ferro posseduta da queste transferrine fa sì che lo stesso elemento non rimanga disponibile per i microorganismi parassiti, in una competizione che è stata suggestivamente chiamata «battaglia degli agenti chelanti» e che rappresenta la base di questo particolare tipo di immunità definita nutrizionale. Occorre però considerare che se da una parte gli organismi superiori sono capaci di sottrarre ferro ai microorganismi tramite le transferrine, gli stessi microorganismi possono a loro volta sintetizzare ed impiegare dei particolari composti a basso peso molecolare, noti come siderofori, in grado di chelare un atomo di ferro per molecola. Sono stati finora riconosciuti in natura circa 200 differenti tipi di siderofori [1] e probabilmente ve ne sono ancora molti da scoprire.

Dal punto di vista chimico i siderofori possono essere però ricondotti fondamentalmente a due differenti classi chimiche: quella dei composti catecolici e quella dei composti idrossamicici.

I composti catecolici possiedono sei gruppi idrossilici mediante i quali possono legare lo ione ferro con un legame di coordinazione. I composti idrossamicici legano invece il ferro con i residui N-O e C-O.

La classe chimica dei catecoli, rappresentata dall’enterochelina, ha una maggiore affinità per lo ione ferro rispetto ai composti idrossamicici, mentre le transferrine si pongono ad un livello intermedio [2].

Occorre ancora notare come nell’ambito dei vari generi e delle varie specie microbiche siano prodotti siderofori di tipo catecolico e/o di tipo idrossamico. La sintesi di questi composti può essere copiata dal cromosoma (in genere per i composti catecolici) o legata a plasmidi (in genere per i composti idrossamicici). In alcuni casi però (Shigella, Pseudomonas) la
La sintesi di siderofori idrossamici può essere codificata dal cromosoma. Qualunque sia il tipo di sideroforo prodotto, la sintesi di esso è in ogni caso regolata in funzione della concentrazione del ferro disponibile nel mezzo ambiente, in quanto alte concentrazioni di ferro all'esterno rendono possibile il trasporto passivo di tale elemento attraverso la membrana cellulare e quindi la sintesi dei siderofori viene repressa. Al contrario, in condizioni di stress di ferro, situazione abbastanza tipica in alcune infezioni, viene indotta la sintesi dei siderofori e dei loro specifici recettori.

Il trasporto del ferro mediante il sistema dei siderofori e dei recettori per questi è rappresentato nel seguente schema che si riferisce ad *Escherichia coli* K 12 (Fig. 1). Da questo schema risulta che la sintesi dei siderofori avviene vicino alla membrana citoplasmatica. Dopo che il sideroforo è stato versato all'esterno si ha il legame con lo ione ferrico e quindi il complesso ione ferrico-sideroforo viene fissato ad uno specifico recettore situato sulla parete cellulare.

Essendo il complesso ferro-sideroforo di dimensioni che superano il limite di esclusione delle porine di *E. coli*, è necessario un sistema di internalizzazione più complesso che involve una proteina codificata da ton B. Mediate questa proteina viene effettuata la traslocazione del complesso ferro-sideroforo ad una permeasi a livello della membrana citoplasmatica. All'interno della cellula il ferro viene infine rilasciato grazie ad una reduttasi.

In effetti nello stesso *E. coli* K 12 il sistema di trasporto del ferro può essere notevolmente più complicato e questo batterio costituisce in proposito un modello molto importante, non soltanto perché si tratta del ceppo meglio conosciuto dal punto di vista genetico, ma anche perché in esso possono essere presenti contemporaneamente differenti sistemi di trasporto del ferro, sia a codificazione plasmidica che cromosomica.

È questo il caso di *E. coli* K 12 portatore del plasmide Col V (Fig. 2) in cui viene codificata la sintesi di tre distinti recettori per gli idrossammati sulla membrana esterna: flu E per il coprogeno, flu A per il ferricromo e iut A per l'aerobactina. Il trasporto alla membrana citoplasmatica del sideroforo ferrico avviene mediante ton B, mentre i prodotti genici di flu C e flu D (e forse di flu B) possono contribuire al distacco dello ione ferrico dal complesso con l'idrossammato.

Il citrato ferrico ha invece come recettore fec A e l'internalizzazione avviene mediante ton B e fec B. L'enterochelina ferrica è legata infine dal recettore fep A sulla parete cellulare e dal recettore fep B che è probabilmente sulla membrana citoplasmatica insieme a ton B.

Un punto interessante a proposito di *E. coli* K 12, ma anche di altri batteri, è come a fianco di siderofori catecolici codificati dal cromosoma vi possa anche essere la presenza di plasmidi che mediano la sintesi di siderofori idrossamici.

Questo fenomeno è stato da noi studiato anche in ceppi epidemicì di *Salmonella wien* e *Salmonella typhimurium* [3] nei quali in particolare è stata identificata la regione plasmidica che codifica sia per la sintesi dell'aerobactina che per il suo recettore clo.

Non è chiaro perché in alcuni batteri patogeni il sistema dell'enterochelina abbia bisogno del supporto del sistema dell'aerobactina.

Se si paragonano in astratto queste due classi di siderofori, va notato che in soluzione tampone i catecoli hanno affinità superiore agli idrossammati per il ferro. Gli idrossammati possono però essere più efficaci in presenza di siero ed a pH sotto la neutralità. Gli idrossammati possono anche superare i catecoli nella solubilità in acqua, nella stabilità, nella capacità di essere riciclati e più difficilmente risultano antigeni [1]. Quest'ultimo punto può avere

![Diagramma](image_url)
Dopo aver esposto alcuni significativi esempi di quanto avviene in batteri patogeni appartenenti a gruppi diversi, è interessante fare alcune considerazioni su quelle specie che in particolare possono agire da patogeni opportunisti nei confronti dell’ospite immunocompromesso. Qui il discorso dovrebbe essere molto ampio ed articolato, perché è noto quanto numerose siano le specie che possono comportarsi da patogeni opportunisti. Purtroppo non per tutte vi è ricchezza di dati e ci sembra quindi più opportuno focalizzare questa esposizione su due specie particolarmente importanti e che rappresentano due situazioni molto diverse fra loro.

La prima è rappresentata da *Pseudomonas aeruginosa* in cui accanto ad un sistema mediato dal citrato, al trasporto del ferro possono concorrere entrambi i tipi di siderofori e cioè la piochelina (catecolico) e la pioverdina (idrossamico). Studi condotti con mutanti privi di uno o entrambi questi tipi di siderofori [7] hanno dimostrato come la presenza di uno solo dei sistemi di trasporto del ferro consenta la moltiplicazione in presenza di sierotransferrina. Ciò dimostra che questi siderofori sono capaci di sottrarre il ferro necessario per lo sviluppo dal complesso sierotransferrina-ferro, mentre in assenza di entrambi i siderofori la crescita è invece drammaticamente arrestata.


L’altra specie cui desideriamo accennare non è una specie batterica, ma quella di un micete, *Candida albicans*, ben noto soprattutto per le infezioni che può dare nell’ospite immunocompromesso.


Ma a proposito di *Candida albicans* un altro interessante dato è emerso da nostre ricerche condotte sull’attività antimicrobica delle transferrine. Abbiamo infatti notato che l’attività antimicrobica dell’ovotransferrina si manifesta sia con l’ovotransfer-
rina insatura che con l'ovotransferrina saturata con ferro [10].
Una simile osservazione induce a ritenere che l'azione anti- *Candida* delle transferrine non sia dovuta al semplice meccanismo di chelazione del ferro ma sia in realtà più complessa.
Una risposta a questo interrogativo è stata data da esperimenti condotti in parallelo in presenza di ovotransferrina insatura ed in terreno povero in ferro ottenuto a mezzo di transferrina immobilizzata su Sepharose 4B ed il cui contenuto in questo metallo libero era praticamente uguale a quello che si aveva per azione della presenza della transferrina insatura [11].
I risultati ottenuti dimostrano che *Candida albicans* riusciva a moltiplicarsi per almeno cinque generazioni in terreno povero di ferro, mentre in presenza di transferrina non si andava oltre le due generazioni.
Questo tipo di esperimento è stato successivamente ripreso [11] coltivando in parallelo *Candida albicans* in terreno di Winge, in terreno povero di ferro, in terreno in cui il ferro era stato chelato per aggiunta di 2-2'-dipiridil, in terreno in cui *Candida albicans* veniva fatta sviluppare separata dalla transferrina a mezzo di una membrana di dialisi, ed infine in presenza di transferrina libera. I risultati ottenuti hanno dimostrato che la maggiore inibizione si aveva sempre quando *Candida* era a contatto con la transferrina.
Questi nostri risultati si ricollegano a quelli ottenuti da Artis *et al.* [12] i quali, usando *Trichophyton mentagrophytes* e *Rhizopus oryzae*, hanno suggerito che l'interazione fra la sierotransferrina e la superficie della cellula fungina sia mediata da un mistero complesso ligando formato dalla sierotransferrina e dai siderofori idrossamici associati alla parete cellulare fungina. Controlli da noi eseguiti con transferrina coniugata con sostanze fluorescenti hanno messo in evidenza un legame della molecola di transferrina alla superficie di cellule di *Candida* e sembrano confermare quindi questa particolare interazione transferrina-cellule. Non possiamo affermare se questo assorbimento interferisce con il metabolismo del ferro o altre funzioni microbiche essenziali, ma in ogni caso questa attività antifungina, alternativa al semplice sequestro del ferro, dà alle transferrine un significato biologico ancor maggiore.

**BIBLIOGRAFIA**


