BioMetals 1997, **10**, 227–236

Abstracts – International Conference on HIV and Iron

Brugge, Belgium – March 14–15 1997 (organized by Dr Johan R. Boelaert)

1.1 IRON CHELATION DECREASES NF-κB AND HIV-1 ACTIVATION DUE TO OXIDATIVE STRESS

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An important aspect of human immunodeficiency virus (HIV-1) infection is the regulation of its expression by nuclear factor κB (NF-κB) through redox-controlled signal transduction pathways. In this study, we demonstrate that iron chelation by deferoxamine (DFO) protects against the cytotoxic and reactivating effects of hydrogen peroxide (H_2O_2). These protective effects were observed both in lymphocytic (ACH-2) and promonocytic (U1) cells latently infected by HIV-1. Concomitantly, NF-κB activation by H_2O_2, when followed by gel retardation assay, was decreased in the DFO-treated U1 and ACH-2 cells. This latter DFO-mediated effect was specific, as DFO did not clearly affect AP-1 DNA-binding activity when studied after H_2O_2 induced stress. More importantly, DFO protected against the H_2O_2-induced activation of HIV-1 as evidenced by reverse transcriptase activity in the supernatant. DFO also protected against PMA-induced NF-κB activation as well as TNF-α-induced HIV-1 activation. Furthermore, DFO attenuated the p24 response in PBMC infected with HIV-1 and stimulated with IL-2. These different effects of DFO were obtained at DFO concentrations lower than 5 μM. Other chemically unrelated iron chelators also provided protection against cytotoxicity, NF-κB activation and HIV-1 activation in U1 cells challenged with H_2O_2.

1.2 IRON CHELATION DECREASES HIV-1 TAT MODULATED TNF-INDUCED NF-κB ACTIVATION IN JURKAT CELLS

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TNF-α stimulates HIV-1 replication via activation of the transcription factor NF-κB. NF-κB activation by TNF-α is known to involve the intracellular formation of reactive oxygen intermediates (ROIs). We recently demonstrated that HIV-1 Tat protein potentiates TNF-induced NF-κB activation by downregulation of Mn-dependent superoxide dismutase (MnSOD), shifting the cellular redox state towards pro-oxidative conditions. This study shows that iron chelation by deferoxamine (DFO) or L_1 : iron chelators of different chemical classes, strongly decreases HIV-1 Tat modulated TNF-induced NF-κB activation in Jurkat cells, but does not modify NF-κB activation by TNF-α. The ability of iron chelators to reduce Tat potentiated activation of NF-κB by TNF suggest that iron and intracellular hydroxyl radicals (OH^o) are required for Tat effect. Moreover, we have shown that exogenous OH^o was capable of potentiating TNF-induced NF-κB activation but by itself was not sufficient to trigger activation of NF-κB. In addition, DFO and L_1 had no effect on MnSOD activity in Jurkat cells or on decreased MnSOD activity by Tat. Iron chelators had also no effect on the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) in Jurkat cells, but have been able to elevate the GSH:GSSG ratio which has been decreased by Tat. Our observations suggest that iron and intracellular OH^o may play a role in the potentiation of TNF-induced NF-κB activation by HIV-1 Tat protein and that iron chelation may protect Jurkat cells, at least in part, against oxidative stress induced by Tat.

1.3 EFFECT OF HAPTOGLOBIN POLYMORPHISM ON THE OUTCOME OF HIV-1 INFECTION

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Haptoglobin (Hp) is a hemoglobin-binding protein with three genetic types: Hp 1-1, 2-1 and 2-2. Hp is an endogenous antioxidant, protecting against hemoglobin/iron driven oxidation. Oxidative stress promotes HIV-replication. In this study, we investigated the outcome in HIV patients according to Hp phenotype. The study group consisted of 620 Caucasian patients (527 males, 93 females) with a proven HIV infection. The average follow-up time was 47 months. In the HIV infected patients, 111 subjects (17.9%) showed a Hp 1-1 type, 308 persons (49.7%) had a Hp 2-1 and 201 (33%) a Hp 2-2 phenotype. The observed Hp frequencies correspond with those of the reference population. Serum Hp concentrations correspond with the Hp phenotype-related reference values and were lowest in the Hp 2-2 patients. Kaplan-Meier curves showed a significantly lower survival time for Hp 2-2 carriers: median survival time 7.17 years vs. 10.75 years for the other Hp types (log rank test $p = 0.0012$). This difference was mainly due to a significantly ($P < 0.05$) shorter period before evolution to the AIDS stadium: 1401 ± 905 days (Hp 1-1) versus 1359 ± 901 days (Hp 2-1) and 1096 ± 931 days (Hp 2-2). The higher mortality for the Hp 2-2 subgroup suggests an effect of iron-mediated oxidative stress on the survival following HIV infection.

3.1 TOXICITY OF SERUM FERRITIN

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Previously, we have demonstrated the toxic role of serum ferritin from thalassemic patients on neutrophil (PMNs) phagocytosis. This dysfunction has been attributed to an excessive generation of oxidant radicals. A clinically important degree of iron overload may be seen in HIV positive patients, mainly in relation with blood transfusion and with zidovudine treatment. High levels of serum ferritin were observed in HIV positive patients reflecting the iron overload but also the inflammatory response of these patients. The purpose of the present study was to investigate the role of serum ferritin, isolated from HIV patients on neutrophil phagocytosis. The serum fraction containing ferritin and no transferrin, albumin and low molecular weight iron compounds was isolated by gel filtration chromatography. We tested in parallel the ferritin extracted from HIV patients and from healthy controls (HC) on PMN phagocytosis from HC. PMN phagocytosis was measured by flow cytometry before and after incubation, in presence of phorbol myristate acetate at priming concentrations (1 ng/ml). The corrective effect of desferrioxamine (DFO) was evaluated. Results were significantly different between incubation with ferritin from healthy controls and from HIV (75% of phagocyting PMN after incubation with normal ferritin vs 61% with ferritin from HIV patients ($p < 0.02$)). DFO corrected the phagocytosis abnormality observed in presence of ferritin from HIV patients ($p < 0.02$). DFO had no effect on phagocytosis after incubation with ferritin from healthy controls.

In conclusion, the ferritin isolated from HIV patients has a toxic effect on PMN phagocytosis, in relation with its iron content. The progression of HIV infection toward its more advanced stages is

accompanied both by increasing serum ferritin levels and progressive phagocytic defect. These two observations could be related, at least partially.

3.2 MECHANISMS OF FERRITIN GENE EXPRESSION IN INFLAMMATION AND INFECTION.

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Inflammatory cytokines induce perturbations of iron metabolism as reflected by increased iron stores in the macrophage/monocyte cell population and elevated serum ferritin levels. It is not yet clear whether the intracellular iron retention results from a primary cytokine-mediated increase in ferritin synthesis or from a higher rate of iron incorporation into cells with a subsequent stimulation of ferritin synthesis. Ferritin gene expression is regulated at both the transcriptional and translational levels. Multiple regulatory elements have been identified upstream of the H ferritin gene which activate transcription in response to various stimuli. Among these, an NF_{κ}-B binding site has been shown to induce H ferritin transcription in response to TNF-α or IL-1, but this effect has only been studied in fibroblasts and adipocytes. Nothing is known about the regulatory elements implicated in L ferritin gene expression, although all the evidence points towards a limited effect of cytokines. In addition, iron-responsive or cytokine-responsive elements are present in the 5' non coding region of both H and L ferritin mRNAs and modulate the rate of ferritin synthesis. However, the relative proportion of H and L mRNA in the pool of untranslated ferritin mRNA governs the subunit composition of the ferritin molecule and the rate of iron uptake by the protein.
To precise the role of iron and cytokines in modulating ferritin gene expression in inflammatory conditions, we have followed changes in NF_{κ}-B DNA binding activity and measured H and L ferritin mRNAs in response to TNF-α addition in T lymphocytic (Jurkatt) and promonocytic (THP-1) cell lines. The role of iron in NF_{κ}-B activation was also studied in stable transfectants of the same cell lines, which overexpress the H subunit and have a subsequent depletion of the labile iron pool. The results will be discussed in relation with the experimental evidence described in the literature.

3.3 HIV-1-INDUCED MODULATION OF CD71 EXPRESSION

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To investigate whether HIV-1 infection affects the expression of the transferrin receptor (CD71), CD4+ cell lines expressing CD71 at different basal levels were infected *de novo* with HIV-1 and tested for cellular surface CD71 expression after 2 and 5 days of incubation. We compared the density of surface CD71 on control uninfected and HIV-1 infected cells. The results obtained indicate that cells acutely infected with HIV-1 express CD71 at lower levels. The extent of this cell phenotypic modulation depends on the nature of the cell lines tested. In HIV-1-infected MT-4 cells, CD71 was reduced by a rate of 62.2% on the 2nd day after infection. On the 5th day, such cultures displayed a broad cytopathic effect amounting to 67.6% dead cells. HIV-1 infected Supt-1 cells had a non-significant decrease of CD71 on the 2nd day after infection, whereas it amounted to 48.6% on the 5th day. In these cells a non-syncytial cytopathic effect of HIV-1 occurred on the 8th day of incubation. In HIV-1 infected myelomonocytic THP-1 cells, surface CD71 was decreased by a rate of only 9.2% on the 5th day after infection: the decrease of CD71 levels was parallel to a differentiation of HIV-1 infected THP-1 cells into macrophages. On the contrary, H9-HTLV III_B cells, a T-CD4+ cell line constitutively infected with HIV-1 express the transferrin receptor at higher levels than control uninfected H9 cells. Furthermore, H9-HTLV III_B cells showed to be more sensitive towards the cytotoxic effects of desferrioxamine as compared to control uninfected H9 cells. On the whole, these data suggest that reduction of cell surface CD71, or reduction of iron uptake, is associated with cell death in HIV-1 infected cultures.

4.1 FERRITIN STIMULATES NO SYNTHASE ACTIVITY AND REDUCES NITRIC OXIDE-INDUCED RNA-BINDING ACTIVITY OF IRP1 IN MACROPHAGES.

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The involvement of nitric oxide (NO) in the intracellular iron metabolism accounts for the inhibition of certain iron-containing enzymes, formation of electron paramagnetic resonance (EPR) - detectable iron-nitrosyl complexes, loss of intracellular iron, as well as the post-transcriptional regulation of ferritin (FR) and transferrin receptor (TfR) synthesis via modulation of iron regulatory protein 1 (IRP1) activity. On the other hand, modulation of the chelatable pool of iron in the cell by desferrioxamine and/or iron salts has been shown to regulate nitric oxide synthase (NOS) activity. Altogether, these data suggest the existence of a regulatory loop between iron homeostasis and nitric oxide metabolism. Here we report that FR, one of the main regulators of intracellular iron homeostasis, stimulates NOS activity and, concomitantly, modulates the effect of NO on the formation of the IRP1-RNA complex. Our results indicate that mouse liver ferritin (MFR) acts synergistically with IFN-γ to induce NOS expression in the murine RAW 264.7 cell line and peritoneal macrophages. This induction was observed at the level of nitrite concentration, NOS activity in cell homogenates as determined by [^{3}H]-citrulline synthesis, and NOS mRNA accumulation analysed by Northern blot. In a parallel study, we compared the RNA-binding activity of IRP1 in RAW 264.7 cytosolic extracts from IFN-γ/LPS and/or IFN-γ/MFR-activated cells, which express similar NOS activity. MFR partially prevented NO from activating IRP1. We therefore tested whether such an effect of FR could be associated with enhanced formation of an EPR-detectable iron-nitrosyl complex bound to FR. Data showed that the EPR <g = 2.04 signal> associated with FR in cytosolic extracts obtained from K562 cells after coculture with IFN-γ/LPS-activated RAW 264.7 macrophages accounted for only 10–15% of the signal emitted by the whole cytosol. Taken together, our results suggest that FR may influence NO metabolism and effects in the cell. This activity of FR may be an important component of the interplay between the intracellular iron metabolism and NO synthesis.

4.2 NITRIC OXIDE AND VIRUSES: AN OVERVIEW

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L-arginine dependent production of reactive nitrogen intermediates (RNIs: nitric oxide (NO), nitrite and nitrate) has been proposed to occur via an L-arginine oxidative deimination pathway and is known to be responsible for certain antineoplastic and antimicrobial effector functions. NO, from both endogenous and exogenous sources, has also been shown to restrict viral replication of certain viruses. Homan monocytes infected with different viruses, including HIV-1, produce modest but significant levels of NO. Serum nitrite and nitrate levels are elevated in individuals with HIV-1 infection, suggesting that cytokine-mediated NO-synthesis also occurs in human HIV infection *in vivo*. On the other hand, regulatory HIV-1 proteins, such as Tat, may modulate interferon-γ specific signal transduction pathways leading to NO-synthase expression.
NO may have protective, as well as injurious, actions. The putative role of NO in the neuropathogenesis of different viral disorders (including those related to HIV-infection) has recently been proposed. NO contributes to the toxicity of the HIV type 1 coat protein gp120 toxicity: human glia respond to gp120 with NO production. Cytoprotection against gp120-induced neuronal cell death can be afforded by inhibitors of L-arginine analogues.
This putative dual action of NO in viral infection in general and HIV-infection in particular will be discussed.

4.3 NITRIC OXIDE AND RETROVIRUSES IN MICE

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Nitric oxide (NO) is a highly reactive molecule produced from a guanidino nitrogen of L-arginine in a reaction catalysed by a family of NO synthase (NOS) enzymes. NO has a wide range of physiological and pathophysiological functions. NO and related reac-

tive nitrogen intermediates exert microbiostatic and microbicidal activity against a broad spectrum of pathogens such as bacteria, parasites and viruses. Recently, a number of studies have indicated that NO has antiviral activity. Studies of NO and murine retroviruses suggest that NO may play a role in defences against Friend leukemia retrovirus (FV). In fact, NO-generating compounds (SIN-1, SNP and SNAP) inhibited FV replication *in vitro*: however, their controls, known as not releasing NO, have no effect. In addition, activated macrophages inhibited FV replication in a dose dependent manner as compared to unstimulated macrophages. This inhibition coincided with the production of significant amounts of nitrite (NO2-). Addition of L-NMMA, an NOS inhibitor, reduced the ability of activated macrophages to inhibit FV replication and to produce NO2-. Furthermore, *in vivo* administration of L-NAME, an NOS inhibitor, significantly increased the viral load in spleen cells of FV-infected mice and resulted in exacerbation of infection. However, we could not demonstrate the role of NO in LP-BM5 infection, another murine leukemia retrovirus, inducing an immunodeficiency syndrome known as murine AIDS (MAIDS). LP-BM5 infection did not increase macrophage NO production and *in vivo* inhibition of NO did not appear to affect LP-BM5-induced immunodeficiency.
Our results suggest that NO is not involved in MAIDS pathogenesis, in contrast to the involvement of NO in the FV pathogenesis. These discrepancies could be related to differences between the two retroviruses which could exhibit different susceptibilities to NO. NO may affect viral pathogenesis through a variety of mechanisms depending on the host and retroviral specificity. The results reported about the effects of NO on HIV infection are also under debate.

5.1 OVERVIEW OF POPULATIONS AFFECTED BY BOTH IRON OVERLOAD AND HIV INFECTION.

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Iron overload has an adverse effect on the body's immune defenses, and it can be postulated that this effect may be magnified in the immune deficiency that accompanies HIV infection. While iron-loading disorders have generally been considered to be rare, it is now known that these conditions are fairly common in certain populations. Dietary iron overload is present in up to 10% of members of rural African communities. Homozygous HLA-linked hemochromatosis is found at a rate of about 5 per 1000 in populations derived from Europe. Thalassemia major and intermedia syndromes, which are associated with iron-loading even in the absence of blood transfusions, are prevalent in southeast Asia. It seems possible that iron overload disorders may put some segments of the population at risk for rapid progression of AIDS.

5.2 BONE MARROW IRON STORES AND SURVIVAL IN PATIENTS WITH HIV INFECTION.

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To determine if there may be a relationship between body iron stores and survival in patients with HIV infection, we retrospectively analyzed bone marrow iron stores and duration of survival in 224 patients with HIV infection who had clinically indicated diagnostic bone marrow biopsies performed. The study group included 13 females and 211 males with a mean (±SD) age of 38 ± 8. Bone marrow iron was graded on a scale of 0 to 6 with 0–1 (n = 23) indicating low iron stores, 2 (n = 60) normal iron stores, 3 (n = 86) normal to increased iron stores and 4–6 (n = 55) markedly increased iron stores. Survival and mortality were assessed by reviewing hospital records and death certificates. The patients were followed for a median of 185 days after the bone marrow study (range 1 to 1520). During this time 66 (29%) of the subjects died. Cox proportional hazards analysis indicated that, after adjustment for age and sex, each 1 point increase in bone marrow iron grade was associated with a 1.4 fold increase in the rate of mortality (95% confidence interval of 1.1 to 1.8, $p < 0.02$). Similar results were found for a subset of 154 subjects in whom sufficient data was available to adjust for the significant baseline variables of cytomegalovirus infection and urinary tract infection. These results suggest that increased storage iron may adversely affect survival in HIV infection. The mechanism is not known, but could conceivably be due to an iron-related impairment of the response of macrophages to TH-1 stimuli.

5.3 BODY IRON STORES AND THE PROGRESSION OF HIV DISEASE

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Introduction: To determine if the magnitude of the body's iron stores is related to the progression of HIV disease, we studied 60 Zimbabwean men who had recently undergone seroconversion.
Methods: The men were recruited from a cohort of 2990 factory workers whose HIV status was checked every 4-6 months. The iron stores of the study subjects were estimated on the basis of serum ferritins determined before seroconversion; body iron stores in mg can be estimated by multiplying the serum ferritin in ng/ml by 10. The subjects were followed for a median of 14 months after seroconversion (range of 1 to 37), and study outcomes were (i) death and (ii) progression to a CD4+ lymphocyte count of less than 100×10^9/l.
Results: The mean age of the study subjects was 29 ± 8 years and preconversion serum ferritins ranged from 1 ng/ml to 343 ng/ml. Study subjects were characterised according to whether serum ferritins were greater or less than 83 ng/ml (the mean value for healthy men aged 20–44 years as determined in a community survey conducted previously (Lancet 1986; 1 : 1310). In the group with higher serum ferritins (n = 22), median estimated iron stores were 1100 mg (range of 840 mg to 3430 mg). In the group with lower serum ferritins (n = 38), median estimated iron stores were 440 mg (range of 10 mg to 810 mg). Statistical analysis was performed with Kaplan–Meier and Cox proportional hazards analyses. Two deaths occurred, both in the group with higher iron stores (Mantel log rank test = 0.026). The occurrence of a sexually transmitted disease (STD) after seroconversion was a significant risk factor for progression to a CD4+ lymphocyte count of less than 100×10^9/l ($p = 0.001$). After adjustment for the occurrence of STD, the relative rate of progression to a CD4 + lymphocyte count of less than 100×10^9/l among patients with higher iron stores was 2.2 times the rate among patients with lower iron stores (95% confidence interval of 0.9 to 5.4).
Discussion: Our results are consistent with the hypothesis that higher body iron stores favor the early progression of HIV disease.

5.4 INTERPLAY BETWEEN HEPATITIS C VIRUS (HCV), HIV AND IRON.

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Co-infection with HIV & HCV frequently occurs, mainly in injecting drug users (IDU) & hemophiliacs. As both HIV & HCV infections may modulate/be modulated by Fe status, we reviewed the literature in order to answer 4 questions.
1) Does HIV alter the course of HCV infection? 7 cross-sectional studies indicate that HIV-posit. pts have usually higher HCV RNA concentrations in blood than HIV-negat. counterparts. After HIV seroconversion, PCR RNA increased by 0.6 logs, 6 out of 7 studies (5 cross-sectional & 2 longitudinal) showed an unusually rapid progression to cirrhosis in HIV-posit. pts.
2) Does HCV alter the course of HIV infection? 3 cross-sectional studies & 1 longitudinal one (the latter over 30 months only) found similar progression rates to AIDS, independently of HCV aerological status.
3) Interplay between HIV and Fe. HIV infection is accompanied by progressive Fe accumulation in several tissues (sometimes including liver). Furthermore, it is likely that this iron loading exacerbates HIV infection. Reviewed by Boelart JR, Weinberg GA, Weinberg ED (Infect Ag Diseases 1996).
4) Interplay between HCV and Fe.
-4a. Liver iron concentration (as assessed by atomic absorption spectrophotometry) is at the upper limit of normal or elevated in HCV infection (3 studies) and is higher in genotype 1b than in 2a or 2b (1 study). Data on the relationship

between liver iron concentration & HCV RNA are scarce. Furthermore, HCV infection leads to free-radical-mediated hepatic lipid peroxidation & to an increase in serum ferritin.
-4b. In thalassemics with non-A, non-B hepatitis, the severity of chronic hepatitis & hepatic fibrosis is related to the degree of Fe accumulation. In pts with HCV, high fibrosis scores were more frequently found in the presence of iron at the same histological site.
-4c. Response to Interferon-α (IFNα) therapy is negatively affected by hepatic Fe concentration: when higher, more non-responders are found (10 studies, n = 465 pts; contradicted by 1 study, n = 55), 1 study indicates that Fe deposition in Kuppfer cells & portal triad macrophages is signif. associated with poor response to IFNα. 1 study reports that IFNα therapy in HCV hepatitis decreases liver iron concentration.
-4d. Effect of phlebotomy (done until iron depletion is reached):
- without IFNα: beneficial effect on aminotransferase level (5 studies).
- prior to IFNα: improved response to IFNα (3 studies, n = 50 pts).
- after IFNα failure: contradictory results (n = 43 pts).

-4e. Effect of deferoxamine: only anecdotal data available.
-4f. Ribavirin, also used in HCV hepatitis, frequently causes hemolysis and may therefore increase liver iron.

5.5 SERUM FERRITIN, DESFERRIOXAMINE AND EVOLUTION OF HIV-1 INFECTION IN THALASSAEMIC PATIENTS

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In order to study the respective role of the mean serum ferritin level and the mean desferrioxamine (DFX) dose on the progression of HIV-1 infection, 49 seropositive thalassaemic patients were studied. The relative role of the mean daily of DFX and the mean ferritin level on progression to stage IV (or death) was tested with a Cox proportional hazards model including known confounding variables. Nine years after seroconversion, 10% of those who had been prescribed more than 40 mg/kg of DFX daily had entered stage IV versus 39% of those who had been prescribed a lower dose. Patients with ferritin level higher than 1935 μg/l entered more rapidly in stage IV than those with a lower level (31% versus 16%). In the multivariate analysis, the ferritin level was found to be an independent predictor of progression of HIV disease while the mean daily dose of DFX was not. Similar results were obtained when death was the endpoint. Our results support the hypothesis which was recently expressed, that iron overload could be associated with a more rapid progression of HIV-1 infection.

5.6 TOXICITY OF IRON SUPPLEMENTS IN HIV.

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We have conducted a retrospective analysis of the randomized study[1] of two anti Pneumocystis carinii pneumonia (PCP) agents in HIV disease in which one group received an unplanned iron supplement. Patients entering this trial were previously untreated and were started on AZT as well as PCP prophylaxis comparing aerosolised pentamidine (AP) to a combined tablet of dapsone and iron protoxlyate (DIP). The first parameter measured was the CD4 count at three months. AZT induced the expected increase in the CD4 count in the AP group but not in the DIP group supplemented daily for 90 days with 30 mg iron as the soluble protoxylate. Other dapsone trials showing the expected AZT CD4 enhancement will be presented. The rapidity of the loss of the AZT enhancement on the CD4 count induced by the small amount of iron ingested suggests that the effect is not due to total body stores but rather to serum iron levels driving viral replication. A conclusion of this analysis is that the effect of serum iron levels on viral replication rate should be examined in both early and advanced HIV disease. For example, to control the viral replication rate, early patients could undertake a vigorous phlebotomy program as shown to be effective[2] in the treatment of Hepatitis C infection. An additional conclusion from this analysis is that iron-fortified vitamins may be deleterious and the effect of such vitamins on the viral load and CD4 level can and should be studied.

1) D. Salmon-Caron *et al.* J Inf Dis 172: p545–64 1995; Letters J Inf Dis 173: p1044–5 1996 and 174: p241–2 1996
2) Hayashi *et al.* J Hepat 22:268–71 1995.

5.7 IS IRON A CO-FACTOR FOR KAPOSI'S SARCOMA (KS)?

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KS is linked to infection by a new herpesvirus, HHV-8, which in turn is prevalent in populations that acquire HIV by the sexual route. Cofactors along with HHV-8 infection must be participating in the pathway to neoplasia because many HIV/HHV-8 coinfected people do not get KS.
Endemic KS was common in peasant farmers in parts of Africa long before the HIV pandemic and showed a distribution that conforms to continental rifts and associated volcanic, kaolin-dominant soils. The clinical and geographical similarities of KS and podoconiosis, a lymphatic obstruction of the legs, led to a hypothesis that soil exposure causes damage to lymphatics and predisposes to KS (Lancet 1993; 342:1348).
Kaolin particles are very small (<2μ) and gain entry to the skin through sweat glands. Their high negative charge permits adsorbtion of cations including iron oxides. Because unbound iron is highly toxic to tissues (producing oxyradicals via the Fenton reaction), the iron-laden kaolin might cause damage to macrophages and lymphatics of the foot and leg of exposed farmers, leading to impaired local immunity to HHV-8.
We have adduced evidence in support of this hypothesis in a case control study of endemic KS in Uganda, using other cancers as a control group. KS patients are more likely to come from areas characterised by kaolinite lateritic soil, to wear shoes less often, and to be exposed to water than controls. Studies of iron levels in tissues and soils are in progress to learn if iron exposure is pathogenic.

6.1 OVERVIEW ON ALTERED ANTI-OXIDATIVE DEFENSE IN HIV INFECTION

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In 1988 and the subsequent years, a series of publications appeared reporting significantly decreased cysteine, cystine and intracellular glutathione levels in HIV infected patients and SIV infected rhesus macaques (1-5). In view of a large body of complementary evidence showing the importance of the glutathione level in lymphocytes (6,7), it was suggested that HIV infected patients may be treated with N-acetyl-cysteine (NAC) or another cysteine derivative (7,8).
An overview will be given including a brief up-to-date account on the functional consequences and the mechanisms that (dys)regulate the cyst(e)ine level in physiological and pathological conditions.

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6.2 COULD AN ALTERED ANTI-OXIDATIVE DEFENCE PLAY A ROLE IN DRUG INTOLERANCE IN HIV INFECTION?

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Treatment of Penumocystis carinii pneumonia with cotrimoxazole (combination trimethoprim and sulfamethoxazole) is complicated by adverse reactions in 40–80% of the AIDS patients compared to less than 5% seronegative patients with other immunodeficiencies. This high frequency of adverse reactions could be attributed to the formation of reactive hydroxylamine metabolites of sulfamethoxazole combined with a deficient glutathione system needed to scavenge these reactive molecules. We compared the urinary excretion of all metabolites of sulfamethoxazole in HIV seropositives and healthy controls and found that only the hydroxylamines were reduced in sereopositives. Instability of this compound in vivo could lead to covalent bindings and hapten formation. Hydroxylamine formation of different sulfonamides was studied as well and it was noticed that not all sulfonamides are hydroxylated to the same extent; possibly sulfametrole has a more favorable toxicity profile. Determination of free glutathione levels in whole blood, plasma, CD14+- and CD4+-1 lymphocytes revealed however no difference between seropositives and healthy controls.

6.3 TAT-MEDIATED OXIDATIVE STRESS SENSITIZES CELLS TO APOPTOSIS

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Apoptosis of lymphocytes in response to a mitogenic stimulus has been proposed as a mechanism to account for the progressive depletion of these cells seen in HIV infection. Oxidative stress, characterized by increased production of oxidants or decreased production of antioxidants will, under the appropriate conditions, cause apoptosis of target cells. The HIV-1 transcriptional activator protein Tat, which plays an essential role in the viral replicative cycle increases oxidative stress via a reduction in cellular Mn-superoxide dismutase (Mn-SOD) activity and RNA levels. In HeLa-*tat* cells, shifts in redox status are exacerbated by reductions in glucose-6-phosphate dehydrogenase (G6PDH) and NADPH levels. Furthermore, HeLa-*tat* cells have decreased glutathione (GHS) and increased lipid peroxidation. Because of the deficiency in Mn-SOD and G6PDH, the cellular ability to respond to additional stresses is compromised. To test whether stresses are additive, HeLa and HeLa-*tat* cells were incubated at 45º C for increasing times and their viability assessed. After a 6 hour heat shock, viability was 100% and 70% for HeLa and HeLa-*tat* cells, respectively. Pre-incubation of the HeLa-*tat* cells with mercaptopropionylglycine (MPG) or with exogenously added SOD restored the heat resistant phenotype, while pre-incubation of HeLa parental cells with the oxidizing agent paraquat sensitized them. HIV-infected cells secrete Tat which is then taken up by uninfected cells. Therefore, we tested its ability to affect redox status at a distance by co-cultivating primary human lymphocytes in the presence of HeLa or HeLa-*tat* cells. When lymphocytes grown in the presence of the HeLa-*tat* cells are exposed to a mitogen, they die by apoptosis instead of proliferating, suggesting that the Tat-mediated alterations in oxidative state may prime the cells for death.

6.4 APOPTOSIS IN AIDS

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T lymphocytes from HIV-infected persons are highly prone to *in vitro* spontaneous apoptosis which is increased following TcR-dependent activation.[1,2] Not only CD4+ T lymphocytes but also CD8, B and NK cells are undergoing excessive cell death. An extensive study on a large cohort of HIV-infected persons supports the hypothesis that the chronic activation of the immune system is the primary cause of the increased susceptibility to apoptosis of patients T cells.[3,5] Priming for apoptosis is also detected *in vivo* and a recent analysis we performed in lymph nodes of HIV-infected patients showed that a high level of tissue trans-glutaminase (tTG), a Ca^{2+} dependent-enzyme involved in the apoptosis process, was detected in a large number of cells including CD4 and CD8 T lymphocytes, macrophages, follicular dendritic cells and endothelial cells[4]. In an attempt to understand the genetic control of apoptosis in HIV infection, the respective contribution of Bcl-2 and Fas molecules was studied[5,6]. An *in vivo* down-regulation of Bcl-2 is detected in patients' T cells, which renders them highly susceptible to spontaneous apoptosis. This decreased Bcl-2 expression is associated with an increased susceptibility to Fas-triggered apoptosis, induced by agonistic anti-Fas mAbs or recombinant Fas ligand. Importantly, experiments performed with lymphocytes from HIV-infected chimpanzees demonstrated that the barely detectable level of apoptosis in these animals was associated with a strong resistance to Fas-induced apoptosis, in spite of Fas expression and Bcl-2 downregulation[7]. The non-pathogenic lentiviral infection is thus associated with the lack of immune activation and normal expression of survival molecules. The relation between apoptosis and cytokine balance alteration was approached. Through a single cell analysis by FACS of cytokine synthesis, the intracellular expression of Th1 (IL-2, IFNγ), Th2 (IL-4, IL-13) and proinflammatory TNFα cytokines by CD4 and CD8 T cells of HIV-infected persons was studied. Rather than a Th1/Th2 alteration, a modification in Th1 cytokines was detected. In addition, a strong relation was found between IL-4 producing cells and apoptosis. Finally the influence of iron on apoptosis will be discussed.

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8.1 IRON HOMEOSTASIS AND MACROPHAGE FUNCTION IN INFLAMMATION

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HIV infection produces changes in iron metabolism which resemble those seen in many chronic inflammatory conditions. These include a reduction in serum (ie transferrin-bound) iron, and accumulation of iron at sites of storage such as the liver and spleen. These changes may help to limit growth of infectious agents associated with HIV-linked diseases. Macrophages play a key role in these events, as they appear to respond to inflammatory stimuli by retaining rather than releasing iron derived from catabolism of aged erythrocytes. Cytokines such as TNFα and IL-1 contribute to this effect, as they can reduce iron release by macrophages, probably as a result of increased ferritin transcription. However, subsequent translation of the ferritin transcripts is controlled by the Iron-Regulatory Protein/Iron-Responsive Element (IRP-IRE) system. There is some uncertainty as to how this system operates in macrophages, since iron is reported to increase both ferritin and transferrin receptor *in vitro*. However, in macrophages in culture, both IRP1 and IRP2 respond normally to changes in abundance of iron. Likewise the role of mediators such as nitric oxide and hydrogen peroxide, which activate IRP(s) to the IRE-binding form(s), is uncertain as this would tend to decrease ferritin translation. Other factors, such as alterations in the regulation of iron release from macrophages, may also be involved.

8.2 RECIPROCAL INTERACTIONS BETWEEN IRON AND T LYMPHOCYTES. RELEVANCE TO HIV INFECTION.

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With the recent discovery of the hemochromatosis gene as a novel HLA gene (Feder et al. Nat. Genet, 1996, 13: 399–408) and the demonstration of a spontaneous iron overload and abnormalities of iron homeostasis in B2 microglobulin knock-out mice (de Sousa et al. 1994, Immunol. Letters, 39: 105–111; Santos et al. 1996, J. Exp. Med 184: 1975–1985) lacking MHC class I expression and

$CD8^+$ cells, the role of the immunological system in the maintenance of iron homeostasis, as postulated earlier has become decisively established. Earlier work demonstrating the effect of iron on selected lymphocyte surface markers (Santos & de Sousa, 1994, Cell. Immunol. 154: 498–506, Arosa & de Sousa, 1995, 161; 138–142) and on the MLR (Bryan et al. 1981, Immunogenetics, 12: 129–135), depending on MHC class I antigens indicates that at least, in vitro, iron load can have a reciprocal effect on selected lymphocyte populations from distinct HLA-A donors. The relevance of these observations to a disease characterised by an imbalance of CD4/CD8 populations whose progression from seropositivity to AIDS has been associated to HLA phenotype is evident. In this paper, we shall review some of the more recent work on HH, demonstrating the relative impact of HLA phenotype and low numbers of $CD8^+$ cells on severity of iron overload (Porto et al., 1997, Hepatology, 25: 397–402).
From the earlier results and the results of the study of lymphocyte populations in HH patients it might be anticipated that HIV-1 seropositive patients with low numbers of $CD8^+$ cells will have a worse prognosis related to opportunistic infections and tissue toxicity resulting from intracellular iron accumulation.

8.3 THE EFFECT OF IRON CHELATION ON CELL-MEDIATED IMMUNE MECHANISMS

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Desferrioxamine (DFO) recently has been shown to have beneficial effects in the treatment of patients with CMV retinitis. Since ganciclovir/foscarnet failed to prevent the progression of the disease in these patients prior to DFO application we hypothesized that the benefits of DFO are due to inhibitory effects of cell-mediated immunity within the tissue. Evidence for immunomodulatory activity of DFO also was reported by others showing improvement of rheumatoid arthritis and graft versus host disease after bone marrow transplantation. To examine the immunomodulatory features of iron chelators we carried out immunological in vitro experiments with DFO and diethyienetriamine pentaacetic acid (DTPA). DFO (10 μM) and DTPA (100 μM) suppressed the mitogen (phytohemagglutinin, PHA) and allogen-induced lymphocyte proliferation (> 90%), the interleukin (IL) 2 receptor expression and the secretion of IL-2. IL-2-stimulated cytotoxicity by CD8+ and CD16+ (LAK/NK) cells was reduced by 100 μM DTPA (> 95%) but not by DFO. The interferon-induced expression of HLA and adhesion molecules on endothelial cells was differentially impaired by DTPA and DFO. Since DFO and DTPA previously were shown to have anti CMV potencies in vitro the additional immunomodulatory effects might be important for the treatment of virus-associated immunopathogenesis e.g. in AIDS patients with CMV retinitis.

8.4 LINKAGE OF CELL MEDIATED IMMUNITY TO IRON METABOLISM

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Iron balance may represent the key for host cells to mount immunosurveillance against invading micro-organisms and tumour cells. Evidence is rapidly accumulating that the control of iron balance during infections and neoplasias is one of the central battlefields deciding about the fate of the disease. We and others could previously demonstrate that increased intracellular iron concentrations decrease the cytotoxic effector potential of macrophages via interferon-gamma mediated pathways, thus increasing the susceptibility towards infections with intracellular pathogens. Furthermore, we could demonstrate that a product of activated macrophages, which plays a crucial role in host defence, namely nitric oxide (NO), regulates cellular iron metabolism by modulating the affinity of iron regulatory protein to RNA stem loop structures, called iron responsive elements, thus affecting posttranscriptional and translational expression for the proteins ferritin and transferrin receptor. In extending this study it was then shown that transcriptional expression of the cytokine inducible form of NO-synthase (NOS) is regulated by cellular iron content of macrophages which provided evidence to the existence of an autoregulatory loop between iron metabolism and the NO/NOS pathway in macrophages, which links maintenance of iron homeostasis with optimal formation of NO for host defence (for review see Weiss et al. Immunol. Today 16:496; 1995).
Based on this knowledge we then investigated whether our in vitro results would also hold true in vivo. In two studies in children with cerebral malaria (in collaboration with V.R. Gordeuk; George Washington University, D.C.; and P.E. Thuma, University of Hershey, PA) we could provide evidence that the beneficial effect of iron chelation therapy towards survival of such patients is likely to be due to strengthening of the TH-1 effector branch of cell mediated immunity involving the NO pathway according to the mechanisms discussed above. These data provide further support to the notion that modulation of cellular iron homeostasis may be an important strategy for successful treatment of various infections affecting cell mediated immune effector function.

8.5 IRON OVERLOAD ALTERS T HELPER CELL RESPONSES TO CANDIDA ALBICANS IN MICE

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Healing and nonhealing patterns of disease can be detected in mice with systemic and gastrointestinal Candida albicans infections. A strong correlation has been shown between disease outcome and the nature of the predominant T helper (Th) cell response: healer mice develop a Th1 response whereas nonhealer mice a predominant Th2 response. In the present study, we investigated the effect of iron overload on susceptibility of mice to C. albicans infection and on the type of Th immunity elicited. Iron overload greatly increased susceptibility to disseminated infection with low virulence C. albicans cells of exogenous origin. The candidacidal activity and the ability to release nitric oxide and bioactive interleukin (IL)-12 were greatly impaired in neutrophils and macrophages from infected mice. $CD4^+$ T cells from spleens of iron-overloaded mice were found to produce high levels of IL-4 and IL-10 and low levels of interferon-γ. Treatment of iron-overloaded mice with the iron chelator, deferoxamine, resulted in the cure of mice from infection, restored the antifungal effector and immunomodulatory functions of the phagocytic cells and allowed the occurrence of $CD4^+$ Th1 protective antifungal responses. These data indicate that iron overload may negatively affect $CD4^+$ Th1 development in mice with candidiasis, a function efficiently restored by therapy with deferoxamine.

9.1 CELLULAR PHARMACOLOGY OF DESFERRIOXAMINE

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Desferrioxamine B (Desferral ®; DFO) is a trihydroxamic acid used in patients with chronic iron overload or for aluminium chelation in some dialysis patients. This substance is not absorbed by the gastrointestinal mucosa and there is no clearcut evidence available on the exact mechanism by which DFO enters the cells nor its complex with iron is released.
To investigate its cell pharmacology, DFO was radiolabelled by reductive methylation of its amino function and characterized as [^{3}H] dimethyl DFO (Me-DFO). The water/octanol partition coefficients of Me-DFO and its iron complex were found similar and not different from those of DFO and its iron complex (FO).
[^{3}H] Me-DFO and Me-FO are taken up by murine J774 monocytes and by human THP1 macrophages (differentiated by phorbol esters) incubated with the substances at 100 μM. The processes first increase rapidly and then partially level off to reach similar accumulation levels. In J774 cells, the accumulation levels are decreased by *ca.* 25% in the presence of 50 μM chloroquine and by *ca.* 50% by a 10 fold exess of DFO or FO, Me-DFO or Me-FO.
Upon reincubation of adherent differentiated THP1 cells incubated for 24h with 100μM [^{3}H] Me-DFO or Me-FO, in fresh medium, the amount of (^{3}H) label remaining associated with the cells falls to 25% of the initial value within 1 h and then remains stable.

Fractionation by differential centrifugation of J774 cells incubated with [^{3}H] Me-DFO for 1.5, 17 or 24h, reveals that from 1/4 to 1/3 of the [^{3}H] label is present in the particulate fraction and about 2/3 in the cytosol (Hody *et al*., this meeting).
These results indicate that the mechanism by which desferrioxamine B or its iron complex could enter the macrophage is complex: simple diffusion across the plasma membrane does not allow to explain the data and facilitated transport and/or endocytosis should also be considered. Cell fractionation data and the effect of chloroquine further indicate a possible localization of part of the cell-accumulated chelator within lysosomes and endosomes.

9.2 SUSTAINED IRON CHELATION THERAPY WITH A HIGH MOLECULAR WEIGHT FORM OF DEFEROXAMINE.

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The iron chelator deferoxamine (DFO) has a short vascular half-life and is associated with acute and chronic toxicities. In order to minimize the toxicity and maintain therapeutic plasma levels, DFO requires infusion pump administration. A novel, high molecular weight form of DFO, produced by chemical attachment of the chelator to hydroxyethyl starch (HES), largely eliminates these problems. HES-DFO may offer advantages for treatment of several iron overload disorders. In addition, HES-DFO has demonstrated efficacy in animal models in which reperfusion injury causes vascular injury. Two single dose clinical trials have been completed with this drug. The first was a double blinded study in normal volunteers, with the highest dose reaching 150 mg/kg of DFO equivalents. The second trial, conducted in thalassemia major and Diamond-Blackfan patients, demonstrated that a single dose of HES-DFO promoted urinary iron excretion exceeding that measured in the same patients receiving similar doses of DFO. In both studies the drug was well tolerated and high plasma concentrations of DFO equivalents were maintained for a week or more following infusion. In normal individuals receiving 50–150 mg/kg, the residual plasma chelator became saturated with iron only after one week or more of circulation. A modest increase in erythropoietin level was noted four days following infusion in the individuals receiving the highest dose (Kling et al., 1996). In summary, a single intravenous infusion of HES-DFO provides a sustained elevated plasma iron binding capacity, thus offering an alternative to infusion pump-based iron chelation therapy.

9.3 OVERVIEW ON ORAL IRON CHELATORS

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Four parameters which are critical for the development of non-toxic orally active iron chelators will be identified: bioavailability, selectivity for iron (III), distribution and toxicity. Each will be discussed in detail. Arguments are presented for the use of bi- and tridentate ligands as opposed to hexadentate ligands. It is difficult to identify a tridentate ligand with a relatively high selectivity for iron (III). The choice is also surprisingly limited for bidentate ligands, 3-hydroxypyridin-4-ones proving to be the outstanding candidates under biological conditions.
Several 3-hydroxypyridin-4-ones have been investigated in man, they are orally active but suffer limitations due to rapid metabolism, inhibition of critically important enzymes and undesirable distribution. In principle these limitations can be designed out of this compound class. For the treatment of thalassaemia, a prodrug concept utilising efficient liver first-pass kinetics appears to hold many advantages. Alternate strategies might prove to be essential for the treatment of viral, bacterial and parasite infections, should iron chelators show potential in the treatment of such infections. Iron chelators have been demonstrated to be of some benefit in the treatment of malaria.
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9.4 OVERVIEW OF IRON CHELATORS: THE PRESENT STATUS OF DEFERIPRONE

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Deferiprone (L1) is the only orally active iron chelator which has demonstrated its ability to stabilize or reduce iron stores in transfused patients with iron overload. To date, well over 1000 patients around the world have been administered L1 to remove iron from the body. Numerous publications, mostly in cohorts of 12-50 patients, have discussed the drug's efficacy as well as the risk of agranulocytosis and other possible side effects, such as arthritis. In 1993 Apotex obtained the world-wide patent rights for the use of deferiprone from BTG in England. Since then, extensive trials have been sponsored in Canada, the USA and Italy, with plans underway for other countries as well. Apotex has just completed a large, multi-center one year trial to determine the safety profile of L1 in 187 patients with thalassemia who are unable or unwilling to use deferoxamine and the investigators will present their results in April '97. Other studies, currently underway or previously sponsored by Apotex, include a long term maintenance group (3–6 yr), a comparative trial with deferoxamine (1–3 yr), a large short term (1–2 yr) maintenance trial and a compassionate use program. It is anticipated that the results from all of these trials will be submitted to Regulatory agencies in North America and Europe this year for approval as an orally active iron chelating agent in patients who are unable to take deferoxamine.

10.1 DEFEROXAMINE AND *PNEUMOCYSTIS CARINII* PNEUMONIA

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Pneumocystis carinii is a fungus causing *P. carinii* pneumonia (PCP), the most common opportunistic infection associated with AIDS. Independent laboratories (Clarkson et al. and Weinberg et al.) found deferoxamine (DFO) to be active against *P. carinii* in vitro and in animal models of PCP. The effective concentration in vitro offered promise of clinical utility, but very high doses were required to treat both rats (1000 mg kg^{-1}) and mice (400 mg kg^{-1}). Considering the short half-life of DFO, an improved dose response was sought by administering DFO to rats via implanted infusion pumps. Using a novel HPLC assay for DFO, plasma drug concentrations were measured. At the end of a three week infusion period, mean steady state plasma concentrations were 2.5 and 1.3 μg ml^{-1} from mean infused doses of 335 and 195 mg kg^{-1} day^{-1}, respectively. When individual animals were compared, the proliferative or trophozoite form of *P. carinii* was completely cleared from the lungs of all animals with plasma DFO $\geqslant$ 1.5 μg ml^{-1} and from 38% of those animals with plasma DFO between 1 and 1.5 μg ml^{-1}. This agrees with Weinberg's culture data showing that 1-5 μg DFO ml^{-1} completely inhibited growth over a 7 day period. Humans achieve a steady state plasma concentration of 4.15 μg ml^{-1} when infused at a rate of 50 mg kg^{-1} and 10 μg ml^{-1} when infused at a rate of 100 mg kg^{-1}. In summary, clinically tolerated doses of DFO should be effective against PCP.
Weinberg examined other iron chelators for activity against *P. carinii.* Some were active in vitro but none other than DFO were active in vivo against PCP. Pharmacokinetic data help explain why DFO may be particularly active against PCP. At 4 hrs, after an intraperitoneal bolus injection of 1000 mg DFO kg^{-1}, control animal lung DFO concentrations had diminished to 0.4% of the peak value of 70 μg DFO g^{-1} lung tissue. At 4 hrs, after the same dose given to animals with PCP, lung concentrations remained high at 41 μg DFO g^{-1} lung tissue, 17% of the peak of 245 μg DFO g^{-1} lung tissue.

10.2 EFFECTS OF IRON CHELATORS ON REPLICATION OF CYTOMEGALOVIRUS

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Several metal chelators were tested for antiviral activity against human cytomegalovirus (HCMV) in cultures of different human cell types including foreskin fibroblasts, endothelial cells and retinal pigmemtal epithelial cells. The most potent agent at a molar level was 2',2'-bipyridine analogue (VUF-8514) inhibiting 50% of HCMV induced plaques at a concentration (EC50) of 50 nM. However, VUF-8514 was hugely toxic for cultured cells. Desferrioxamine (DFO) and diethylenetriamine penta acetic acid (DTPA) represent two metal chelators with similar antiviral activity and relatively low toxicity. EC50 for DFO and DTPA ranged from 6 to 10 μM for different HCMV strains. Antiviral effects of DFO were completely eliminated by co-incubation with stoichiometric amounts of Fe^{3+} whereas Fe^{3+} influenced effects of DTPA only moderately. DFO and other metal chelators had no effects on immediate early (IE) phase of virus replicative cyclus while they influenced late phases of virus replication. In cultures of retinal pigmemtal epithelial cells induction of oxidative stress by H202 was associated with increased expression of HCMV IE proteins. These effects were suppressed by treatment with DFO but not with ganciclovir or foscarnet. Since the *in vitro* effects of DFO were observed at concentrations that are achievable *in vitro* we tested DFO effects in AIDS patients with HCMV retinitis. DFO at a daily dose 1 g/i.v. inhibited progression of retinitis in patients (n = 4) who were resistant to treatment with ganciclovir and/or foscarnet. These results suggest that metal chelators are potent anti-HCMV agents with ability to suppress processes leading to virus activation. Their clinical testing patients with HCMV disease warrants further attention.

10.3 EFFECT OF IRON, DESFERRIOXAMINE AND CHLOROQUINE ON EXPERIMENTAL MURINE TUBERCULOSIS.

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Data on the effect of iron on tuberculosis (TB) are limited. Kochan (1973) showed that serum tuberculostasis was achieved by unsaturated transferrin. More recently, Gordeuk reported that spleen siderosis was a risk free factor for death from TB (Blood, 1996). Here we studied the impact of iron (Fe) loading, Fe chelation by desferrioxamine (DFO) and cellular iron depletion by chloroquine (CQ) on the multiplication of *M. tuberculosis* in a mouse model of chronic, non-fatal infection. During the 2 weeks before infection, Swiss female mice aged 4 weeks received either no treatment (no R/), 6 × weekly 40 mg/kg CQ by gavage or each other day DFO 100 mg/kg or Fe hydroxide polymaltose 50 mg/kg i.p. Then, the 85 mice were infected i.v. with 2.6×10^4 *M. tuberculosis* CFU, strain H37Rv and treatments were continued up to day 28 (CQ idem, DFO and Fe 2 X/ week). Isoniazid (INH) 25 mg/kg daily from day 1 to 28 was used as positive control. On day 1 after infection, 10 untreated control mice and on days 7 and 28, 5 mice from each group were sacrificed: spleen weight, CFU counts in lungs and cytokine patterns from spleen were assessed. Spleen cell IL-2 and IFN-γ production in response to PPD was assessed by bio-assay.

Results at day 28:	No R/	INH	Fe	DFO	CQ
No CFU (avg+SD)	5.9+0.4	0.5	6.2+0.2	5.6+0.4	5.4+0.2
IFN-γ (IU/ml)	58	5	16	98	32

In conclusion, Fe loading slightly increased and both DFO and CQ slightly decreased multiplication of *M. tuberculosis* in a chronic model of infection. Due to the small No of mice per group (n=5), these changes were N.S. vs control (however, $P < 0.05$ for Fe vs DFO or vs CQ). Splenic IFN-y response was enhanced by DFO but decreased by Fe. Overall, the results suggest that Fe modulates murine TB, either directly or via immunological effects.

10.4 THE IMPORTANCE OF IRON-WITHHOLDING IN *MYCOBACTERIUM AVIUM* INFECTION: *IN VITRO* STUDIES

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Mycobacterium avium is a facultative intracellular pathogen that survives and proliferates inside the macrophages of infected hosts. While rarely infecting immunocompetent individuals, it is the most common systemic bacterial infection affecting AIDS patients. The growth of mycobacterium in general is known to depend on the concentration of iron in the media and different iron chelators were shown to inhibit the growth of *M. avium* in axenic medium. The growth of *M. avium* inside cultured human macrophages is also affected by the presence of the iron-binding protein apo-transferrin.

We infected murine bone marrow derived macrophages (BMMØ) with a highly virulent strain of *M. avium* in the presence of six different iron chelating compounds and measured the intracellular growth of the bacterium for 7 days. Within non-toxic concentrations, deferoxamine and N,N'bis(2-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid (HBED) showed an inhibitory effect. We investigated the role of iron-withholding in the mycobacteriostatic effect induced by IFNγ on the BMMØ. Macrophages were infected with *M. avium* and treated with 100U/ml/day of recombinant IFNγ, which resulted in a strong inhibition of the intracellular growth of the bacterium. However, when the same cells were cultured in the presence of an excess of iron, either in the form of ferric ammonium citrate or holo-transferrin, the mycobacteriostatic effect induced by IFNγ was abolished. These results confirm that the growth of *M. avium* inside their natural host cell is strongly dependent on the concentration of iron available in the cell and suggest that one of the defense mechanisms induced by the immune system in the macrophage is the sequestration of iron. We suggest that these factors should be taken into account when planning a therapy against *M. avium* infection, particularly in the case of AIDS patients.

S. Gomes receives the PRAXIS XXI fellowship BD/3284/94 of the Portuguese Research Council.

10.5 EFFECT OF IRON CHELATORS ON *LEISHMANIA* GROWTH

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Leishmania are obligate dimophic intracellular protozoan parasites that cause a group of diseases of major health and socio-economic importance, ranging from self-healing cutaneous lesions to severe visceral leishmaniosis (VL) or Kala-Azar with a high fatality rate. In immunocompetent patients, *Leishmania infantum* provokes a spectrum of diseases, ranging from simple skin lesion to severe visceral leishmaniosis (VL). In HIV-positive individuals, leishmanial infection causes exclusively visceral disease. In southern Europe it is calculated that between 25% and 70% of adult VL are related to HIV and 1.5-9% of AIDS cases suffer from newly acquired or reactivated VL (WHO report, 1995). Efforts focused on the determination of a treatment to control leishmanosis, are intensified.

Towards this direction and based on our findings demonstrating that *Leishmania* possesses a transferrin receptor (Voyiatzaki and Soteriadou, J Biol. Chem. 1990, 1992) and that iron chelators (DFO, L1 and CP94) reduce the rate of promastigote growth *in vitro* (Soteriadou *et al.* J Antimicrob. Chem. 1995), we have investigated the role of iron on the intracellular growth of *Leishmania* in macrophages. It was demonstrated, using an iron chelation approach, that DFO inhibited parasite growth within macrophages in a concentration dependent manner. FeNTA reversed the inhibition of parasite growth by DFO. We suggest that iron depletion may be an effective mechanism against leishmaniosis, which is considered as an opportunistic infection in HIV-infected individuals.

11.1 SMOKING, IRON AND OXIDATIVE STRESS IN THE LUNG.

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The exact mechanisms for the adverse effects of smoking on the lungs have not been entirely elucidated, but they most probably include oxidant injury. Indeed, on the one hand, cigarette smoke contains a substantial number of oxidants and pro-oxidants and, on the other hand, the cell and tissue damage caused by inhaled smoke results in inflammation, which is itself a source of increased oxidants.

Cigarette smoke is a rich source of oxidants, which are present in both the tar component and in the gas phase. The specific role of iron in the toxicity of cigarette smoke has not been elucidated, but

several arguments plead for a possibly important contribution of iron in the development of pulmonary neoplasia and chronic obstructive lung disease. The alveolar macrophages of cigarette smokers and the lung lining fluid obtained from cigarette smokers contain substantially more iron than specimens from nonsmokers. In addition, alveolar macrophages from smokers release more iron than those from nonsmokers. The source of the increased iron in the lungs of smokers is unknown, but each cigarette contains about 0.042 μg of iron (in addition to other possibly significant metals). It is also possible that cigarette smoke disturbs the balance between bound and biologically active iron.
Inflammation in the lungs and airways of smokers is mainly characterized by increases in the numbers of polymorphonuclear neutrophils and aveolar macrophages, with the latter cells being more activated than those of nonsmokers. The neutrophils and activated macrophages contribute to the increased oxidant burden of the lungs by releasing activated species of oxygen. As a consequence of the higher oxidant burden, it also appears that the lungs of smokers contain higher anti-oxidant defense, particularly in the epithelial lining fluid. B. Nemery is holder of the "Dr. P. Tuytens Leerstoel in Toxicologie"

11.2 THE EFFECT OF CHLOROQUINE (CQ) ON AIDS-OPPORTUNISTS, *CRYPTOCOCCUS NEOFORMANS* IN PARTICULAR

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CQ has been shown by others to exert an inhibitory effect on the following AIDS-opportunists in vitro: *Histoplasma capsulatum* infecting human macrophages (MO) (Newman, JCI 1994). *Legionella pneumophila* infecting human monocytes (Byrd, JCI 1991), *Salmonella* spp. infecting different animal cell lines (Finlay, Biochimie 1988) and *Mycobacterium tuberculosis* infecting human MØ (Crowle, II 1991). Furthermore, CQ exerts a modest effect against *Pneumocystis carinii* (Queener, J Medicin Chem 1995). The only one of these microorganisms for which CQ has been studied in vivo is *H. Capsulatum*: CQ protected mice from fatal infection. For both *H. capsulatum & Legionella*, the beneficial effect of CQ was ascribed to limitation of availability of iron due to CQ accumulation in acid vesicles (endosomes/lysosomes) and hence interference with acid dependent iron release from endocytosed transferrin into the cytosol.
We studied the effect of CQ on *Cryptococcus neoformans* (C.n) both in vitro & in vivo. 1) IN VITRO. The BV-2 mouse microglial cell line, infected by C.n. (ATCC 11240) at a 10/1 ratio, showed enhanced anti-C.n. activity at 8 & 24 h, when preincubated for 1 h with CQ at 10 μM ($P < 0.01$). This effect was further strengthened when C.n. was opsonized. The CQ effect was also mimicked by another weak base (NH4Cl at 10 mM) or by bafilomycin Al (at 300 μM), an inhibitor of vacuolar-type ATPases. Iron nitriloacetate, an iron salt that remains soluble at neutral pH, was used to test whether the CQ effect was due to iron depletion; at 50 μM, FENTA did not abrogate the CQ effect, suggesting that the beneficial anti-C.n. effect of CQ was not due to iron limitation. 2) IN VIVO C57BL/6 mice were injected intracerebrally (i.e.) with C.n. on day 0. In the CQ treatment group, CQ was administered i.e., both 24 and 3 h prior to lethal infectious challenge. Mean survival time was 20 days in the control group versus 48.5 days after pretreatment with 1 μM CQ($P < 0.01$) Likewise, 1 μM CQ pretreatment resulted in a reduction ($P < 0.01$) in yeast growth in the brain (2-fold at days 3 & 7: 3-fold at day 10).
We therefore report on the beneficial effect of CQ against C.n., a major AIDS-opportunist, both *in vitro* and in a model of murine encephalitis after local i.c. injection. As opposed to the effect reported in infection with *Histoplasma & Leglonella*, the anti-C.n. effect of CQ does not seem to be related to iron metabolism. Several other potential mechanisms have therefore to be explored. We and others did not find that CQ influenced the synthesis of reactive nitrogen intermediates. It is possible that CQ exerts an immunostimulating effect, as we found by RT-PCR analysis that brains from CQ treated mice showed induction of TNF-α & IL-6 gene expression. This needs further confirmation and extension to other models of cryptococcal infection. These results are promising in view of: a) the limited cost and toxicity of CQ; b) its wide availability; c) the fact that its derivative hydroxy-CQ has been shown to have beneficial effects in HIV-infected patients (Sperber 1995).

11.3 EFFECT OF HYDROXYCHLOROQUINE ON HIV-1 INFECTED PATIENTS

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Hydroxychloroquine (HCQ), an anti-malarial used to treat patients with autoimmune diseases, has been shown to suppress HIV-1 replication in vitro in T cells and monocytes by increasing endosomal pH which inhibits post-transcriptional production of gp 120. These in vitro observations have been expanded into an in vivo study of HCQ as a potential anti-HIV-1 agent in HIV-1 infected patients. An initial randomized, placebo-controlled clinical trial was conducted in 40 asymptomatic HIV-1 infected patients who had CD-4+ counts between 200 and 500 cells/mm^3. Patients received either HCQ 800 mg/day or placebo for 8 weeks. Virologic or immunologic parameters including HIV-1 PCR, viral culture, antigen and mitogen responses, and proinflammatory cytokines were measured at the beginning and at the end of the study. The amount of recoverable virus declined significantly in the HCQ group over the 8-week period ($P = 0.022$), while it increased in the placebo group. There was no difference in the absolute CD-4 count for either group (HCQ, 262.8 ± 166 cells/mm^3 vs 251 + 163 cells/mm^3 vs placebo, 312 + 121 cells/mm^3 vs 321 ± 124 cells/mm^3). Mitogen and antigen responses remained constant in the NCQ group while T cell proliferative responses to *Candida* decreased in the placebo group ($4.8 \pm 3.6 \times 10^3$ Stimulation Index (SI) vs $3.0 + 3.0 \times 10^3$ SI, $P = 0.032$). Lastly, serum IL-6 levels declined in the HCQ group (14.3 ± 3.5 U/ml vs 12.0 + 16.7 U/ml) but not in the placebo group (11.3 ± 8.8 U/ml vs 7.0 + 11.7 U/ml); this was coincident with a decrease in serum IgG (2563 ± 1352 mg/dL vs 2307 ± 1372 mg/dL, $P = 0.032$) compared with the placebo group (2733 ± 1473 mg/dL vs 2709 ± 1501 mg/dL). A second 16 week clinical trial was conducted in 72 asymptomatic HIV-1 infected patients with CD-4+ counts between 200 and 500 cells/mm^3 comparing the efficacy of HCQ with Zidovudine (ZDV). Preliminary analysis from patients that have completed the study revealed that HCQ and ZDV reduced the amount of recoverable HIV-1 RNA in plasma over the 4 month study period ($P = 0.020$ and 0.001, respectively). There was no difference in CD-4+ counts in both groups before and after the study. HCQ thus may be useful in the treatment of patients with HIV-1 infection.

P.A 6 HIGH PREVALENCE OF IRON OVERLOAD IN A POPULATION WITH HIGH HIV PREVALENCE

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OBJECTIVE: The aim of the study was to determine if iron overload exists among the urban residents of Harare, Zimbabwe, who are known to have a high prevalence of HIV.
METHODS: The study was a cross-sectional postmortem study, involving subjects aged above 16 years and who experienced traumatic, accidental or suicidal death in Harare, Zimbabwe. The subjects were enrolled in the study in an unselected manner but not all subjects undergoing postmortem examination were included. At autopsy, pieces of tissue were collected from the liver, spleen, heart, lungs and skin and stored separately in formalin until analysis of tissue iron concentration. The dry-weight non-haem iron concentration was determined using the method of Torrance and Bothwell. An hepatic iron index was derived by dividing the hepatic hon-haem iron concentration in micromoles/gram dry weight by the age in years.
RESULTS: A total of 135 accident, trauma and suicide victims and 59 hospital patients were studied. Males outnumbered females by about 4:1. Mean tissue iron concentrations were similar between males and females and between accident, trauma and suicide victims and hospital patients. Non-haem hepatic iron concentrations were normal (<30 micromoles/g dry weight) in the majority of individuals. Elevated hepatic iron concentrations in excess of 30 mol/gram were found in 23% of males experiencing accidental deaths. Substantial iron overload (hepatic iron concentration in excess of 180 mol/gram dry weight) was found in 3% of males with accidental deaths. When subjects were classified according to the hepatic iron index, 9% of the males with accidental death had an index equal to or above 1.9, a level that is

comparable to that found in Caucasian homozygotes for HLA-linked haemachromatosis.
CONCLUSION. This study gives a prevalence of iron overload in young urban dwellers suffering accidental deaths that is much higher than the estimated prevalence of 0.45% in HLA-linked haemachromotosis in the West. The prevalence of HIV in the same population is estimated to be in excess of 40% making the interaction between iron overload and HIV infection an important public health issue.

P.A 10 AFRICAN IRON OVERLOAD AS A RISK FACTOR FOR PULMONARY TUBERCULOSIS

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OBJECTIVE; The aim of the study was to determine if iron overload in Africans is a risk factor for the development of pulmonary tuberculosis.
METHODS: The study was a prospective case control study in which cases where sputum-positive tuberculosis patients who presented to a small rural Zimbabwean hospital, and controls were age and sex-matched subjects from the surrounding community. All subjects were screened for the presence of HIV infection. Serum ferritin and transferrin saturation levels were used to assign iron status: in patients with tuberculosis these levels were determined 12 weeks after the start of therapy to permit inflammation to subside. Individuals with an elevated serum ferritin in combination with a transferrin saturation >50% were taken to have iron overload. Individuals with a normal serum ferritin were taken to have a normal iron status. In persons with an elevated serum but normal transferrin saturation, iron status was not assigned.
RESULTS: Of 31 cases and 31 controls studied, the iron status could be assigned in 19 pairs. The ages ranged from 20 to 79 years with a median of 32. The male to female ratio was 7 to 12. Thirteen (68.4%) of the cases were HIV positive compared to 3 (15.8%) of controls while 3 (15.8%) of cases were iron-loaded compared to 1 (5.3%) of controls. A logistic regression model was constructed that included HIV status and iron status. The odds for tuberculosis in subjects with iron overload was 1.7 (95% C1 of 0.1 to 26.4) times the odds in those without iron overload. The odds of tuberculosis in HIV positive subjects was 10.9 (2.2 to 53.2) times the odds in HIV negative subjects.
CONCLUSION: In this small study there was a strong association between pulmonary tuberculosis and HIV seropositivity but the trend for an association with iron overload was not significant.

P.B.1 PILOT STUDY OF DEFEROXAMINE (DFO) IN HIV-POSITIVE PATIENTS

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Our group had studied the effect of DFO and other iron chelators on the H_2O_2-induced reactivation of HIV-1 in HIV-1 latently infected cell lines (U1, macrophage-like; ACH-2, T-lymphocytic) (Sappey et al., AIDS Res 1995). We had shown that DFO, even at low concentrations (<5μM), abrogated following effects of H_2O_2-generated oxidative stress: cytotoxicity; activation of nuclear factor kappa B; activation of HIV-1 transcription, as evidenced by the appearance of reverse transcriptase in the supernatant. This DFO effect was mimicked by other chelators, such as L1, CP94, HBED, 2, 3-DHB, as well as the "reversed siderophores" RSF-leu & RSF-ileu. DFO also attenuated the p24 response in PBMC infected with HIV-1 and stimulated with IL-2.
In view of these promising in vitro results, we undertook in 1994-1995 a pilot study of DFO therapy in HIV-positive patients (pts). 10 stabilized pts with CD4 <300/mm^3 consented to the study. Design: placebo-controlled, doubleblind, cross-over (with 7 days of wash-out). DFO (40 mg/kg/day) or placebo (saline) were given by the SC route for 10h at night during 7 consecutive days. CD^4 count, plasma HIV-1 viral load (PCR, Amplicor) & iron concentration in 24h-urine were measured before and immediately after each of both 7-day treatment periods. Pts characteristics at baseline: male sex 10/10, age: 28–63 (mean 43.5); antiretroviral R/: AZT in 5, ddC in 1, ddl in 2, AZT + ddl in 1, none in 1; serum ferritin 55-649 μg/L (mean 301); CD4 count: 35-369/mm^3 (mean 191); HIV-1 RNA copies/mL (log): 3.3-6.3 (mean 4.7). Mean changes between the end and the start for each of the 2 treatment periods of 7 days each:

	DFO	placebo	P
log HIV-1 RNA copies	–0.14	–0.06	*N.S.*
CD4 count	–26.5	+3.3	*N.S.*
urinary μg iron/mg creatinine	2.17	0.02	

In 3 out of the 10 pts, SC infusion of DFO was accompanied by a slight to moderate local erythema and pain.
In conclusion, the administration of DFO for 7 days at 40 mg/kg/day in stable patients with a CD4 count of about 200/μL did not significantly affect the HIV-1 plasma load and the CD4 count. As predicted, DFO increased significantly urinary iron excretion. Cytokines are currently being assayed on plasma samples.

P.B 3 IN VITRO EFFECT OF IRON CHELATORS ON MYCOBACTERIUM AVIUM

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M. avium complex (MAC) has a growing impact as AIDS-opportunist. It infects macrophages, often iron-replete in the advanced HIV infection stage. The growth of MAC may be dependent on the iron concentration in its surrounding. Indeed, Douvas et al. (1993) reported on the growth-inhibitory effect of serum, due to the iron-withholding activity of serum apotransferrin. Conversely, Dhople et al. (1994) found that feeding mice with a high-iron diet aggravated experimental MAC infection. Iron depletion might therefore be one of the strategies to combat MAC. We studied the in vitro growth-inhibitory effect of 3 iron chelators; deferoxamine (DFO); deferiprone (L1); N.N'bis(2-hydroxybenzyl) ethylenediamine-N,N'diacetic acid (HBED).
1) Axenic medium ("basal defined medium") at pH 6.5 and 3.7°. Growth of MAC (ATCC 25291) was evaluated after 15-25 days by both a semiquantitative visual and a CFU method. MIC50 values (in μM, mean) are for DFO: 16 (visual), 19(CFU); for HBED: 33 (visual), 43 (CFU); for L1:>100 (CFU & visual). Iron saturation of both DFO & HBED abrogated the observed growth-inhibitory effect.
2) MAC infection of J774 murine macrophages in RPMI + 1% FCS at 37°. After 4h of phagocytosis, either DFO at 10 or 50 μM or HBED at 50μM was added to new medium. Intracellular MAC were counted (Ziehl-Nielsen stain) after 3 & 7 days resp. Results give the % macrophages without or with ⩾ 1 MAC.

	Day 3		Day 7		
	control	DFO 50μM	control	DFO 50μM	HBED 50μM
0 MAC	11%	37%	44%	70%	80%
⩾ 1 MAC	89%	63%	56%	30%	20%

In conclusion, both DFO and HBED were found to suppress MAC growth in vitro, when studied both axenically and in the J774 macrophage. Confirmation with human macrophage studies would be desirable.

P.B 11 EFFECT OF THE COMBINATION OF SEVERAL ANTIVIRAL COMPOUNDS AND DESFERRIOXAMINE ON HERPESVIRUS REPLICATION

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Herpesviruses are important opportunistic pathogens in AIDS patients. Herpes simplex (HSV) and varicella-zoster virus (VZV) are mostly responsible for mucocutaneous infections, whereas cytomegalovirus (CMV) is the major cause of retinitis in these patients. Here we present the results of our *in vitro* studies on the combination of different antiherpetic antiviral drugs with the iron chelator desferrioxamine. The IC_{50} of desferrioxamine for CMV ranged from 10 to 40 μg/ml if the infected cells remained in continuous contact with the drug. Pretreatment of the cells with desferrioxamine 24 h prior infection with CMV did not protect the cells from virus infection. Combinations of desferrioxamine with specific anti-CMV drugs were evaluated for their anti-CMV activity, and their mode of interaction (synergistic, additive or antagonistic) was assessed by the isobologram technique. Desferrioxamine and ganciclovir showed synergistic anti-CMV activity, while cidofovir, foscavir and adefovir showed additive activity when combined with desferrioxamine. These drug combination studies are now being extended to HSV infections, as well as iron chelators other than desferrioxamine.