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Protection of mice from malaria after co-administration of recombinant mouse granulocyte-macrophage colony- stimulating factor and methionine-enkephalin

Abstract

The protective effect of co-administration of recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF) and synthetic peptide met-enkephalin (M-ENK) against blood-induced Plasmodium berghei infection in Swiss mice was investigated. Mice co-administered with rmGM-CSF (10.0

mug/kg) and M-ENK (2.0 mg/kg) x 3/day, i.p., beginning on day -1 and continuing through day +4 after the initiation of infection, showed significant suppression ($p < 0.05$) (sometimes even complete elimination) of parasitaemia compared to vehicle-treated controls. However, when administered separately, neither of these agents induced any detectable protective effect. Surprisingly, mice similarly co-administered with rmGM-CSF (10.0 mug/kg) and higher doses of M-ENK (10.0 mg/kg), showed no protection. Polyclonal neutralizing (100%) antibody to rmGM-CSF abrogated the combined protective effect of these agents. Additionally, naloxone (10.0 mg/kg/day x 6, i.p.), a non-selective, opioid receptor antagonist, also blocked the combined protection. Mice that survived the challenge showed a significant increase ($p < 0.05$) in total circulating leukocytes counts, and the pool-size and the phagocytic activity of both the peritoneal and splenic macrophages, ex vivo. Silica (3.0 mg/mouse, i.v.) abrogated the combined protective effect of rmGM-CSF and M-ENK. These results indicate that co-administration of rmGM-CSF and dose dependent quantities of M-ENK in P. berghei-infected mice can protect against malaria, apparently through macrophage-mediated mechanisms.

Full text

INTRODUCTION

Malaria continues to be a major public health problem world-wide. Currently, nearly 2.4 billion people in 100 countries are at risk of malaria, and an estimated 1.5-2.7 million deaths and 300-500 million new malaria cases occur annually [1]. The main victims of malaria are children under the age of 5 years, and pregnant women. The global malaria situation is worsening with each passing day due to various reasons, including global warming which is leading to the spread of malaria to parts of the world where it did not previously exist. It is acquiring dangerous new dimensions as reflected by the increase in the incidence of case of *Plasmodium falciparum* malaria, the parasite responsible for more than 95% of deaths attributed to malaria. The emergence of parasite strains and vectors resistant to anti-malarial drugs and insecticides, respectively, has further compounded the problem. Furthermore, despite over 21 years of effort, a suitable malaria vaccine is not yet available. Biotherapy of malaria [2,3], a promising new approach for the treatment and control of malaria, is however still very much in its infancy.

Mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 23 kDa glycoprotein hormone, which regulates the differentiation and proliferation programme of the committed progenitor cells for granulocytes and macrophages *in vitro* [4, 5]; *in vivo* it promotes cell survival and stimulates hematopoiesis [6]. Additionally, GM-CSF regulates the activation status of the mature macrophages [7-12] and plays an important role(s) in host defense [13]. Peritoneal macrophages from transgenic mice producing high levels of GM-CSF, have been reported to show enhanced anti-bacterial [14] and other functional activities [15]. The complementary DNA and genomic DNA clones of mouse GM-CSF have been isolated [16], and large quantities of recombinant GM-CSF can now be produced with biological properties similar to the native purified molecule [17]. Mature mouse GM-CSF consists of a single polypeptide chain of 124 amino acids (a.a.), with a leader sequence of 17 a.a., and the two disulfide bonds provide the molecule with a 3-dimensional, biologically active configuration. The carbohydrate content of the GM-CSF is not required for biological activity *in vitro* or *in vivo*. CSFs are very potent molecules which are active at picomolar concentrations, and the levels of constitutively produced GM-CSF are very low; nevertheless, its production is rapidly increased during infections [18]. However, not much is known about the role of CSFs during malaria. Two peaks of serum CSF activity have been reported during fatal and non-fatal murine malaria [19], and maximum serum CSF activity was observed just before the attainment of the peak of the circulating leukocyte counts, and the onset of the decline of *P. cynomolgi* infection in monkeys [20]. Later, plasmodial antigens and parasitized-erythrocytes [PE] were demonstrated to induce the production of CSFs by macrophages *in vitro* [21]. Recently, a plasmid encoding murine GM-CSF has been reported to increase protection conferred by a rodent malaria DNA vaccine [22, 23].

Enkephalins, the two endogenous pentapeptides from the brain, with potent opiate agonist activity [24], are known to exert immunomodulatory effects, both *in vivo* and *in vitro* [25, 26]. Methionine-enkephalin (M-ENK) has been reported to modulate the lipopolysaccharide (LPS)-induced production of cytokines [27], and to enhance various immune parameters in AIDS and cancer patients [28]. Additionally, M-ENK and its synthetic congener (Tyr-D-Ala-Gly-Me-Phe-Gly-NH.C_3H_7-*iso*) can biphasically modulate the *in vitro* production of plasmodial antigens-induced CSFs by macrophages [29], and of concanavalin A-stimulated production of lymphokines by mouse splenocytes [30]. Furthermore, we have reported earlier that dose dependent quantities of morphine can protect mice

against fatal *P. berghei* infection [31], and modulate the plasmodial antigen-induced production of CSFs by macrophages, *in vitro* [32]. Nevertheless, the role of enkephalins during malaria remains elusive. Herein, we report that recombinant mouse GM-CSF (rmGM-CSF) and M-ENK co-administration can protect mice against blood-induced *P. berghei* infection, apparently through macrophage-mediated mechanisms.

MATERIALS AND METHODS

Mice

Swiss mice (20 ± 2 g) of both sexes were obtained from the Central Animal Facility of the Institute. The mice were maintained at 22-24° C, and were provided with standard animal feed and clean water, *ad libitum*. All studies were carried out in accordance with the Guide for Care and Use of Animals in Scientific Research, INSA, New Delhi, as adapted and promulgated by the Institutional Animal Ethics Committee.

Parasite

Plasmodium berghei (NICD, New Delhi strain), obtained from Central Drug Research Institute, Lucknow, India, is being maintained by cryopreservation and animal passage. Mice were infected by inoculating with 1×10^4 PE, intraperitoneally (i.p.). The parasitaemia was monitored by examining 1×10^4 erythrocytes in Giemsa-stained, thin blood films, and expressed as percentage PE.

Drugs, reagents and injection schedule

rmGM-CSF and M-ENK, and rabbit anti-rmGM-CSF polyclonal antibody (IgG fraction) were purchased from Sigma, USA, and stored at - 20° C and 4° C, respectively. Mice (6/group) were injected i.p. with 0.2 ml sterile 5.0% normal mouse serum/saline (vehicle) containing 0.312, 1.25, 5.0 and 10.0 mug/kg rmGM-CSF and/or 2.0 and 10.0 mg/kg M-ENK, three-times daily at 8:00 A.M., 4:00 P.M. and 10:00 P.M. All observations and analyses were commenced at 9:00 A.M. on the next morning following the last injection. Control mice were untreated or received vehicle only. Rabbit anti-rmGM-CSF antibody was dialysed extensively, and a working dilution (1:2,500 in PBS 0.01 M, pH 7.2) was prepared, filter-

sterilized (0.2 mum), and injected into each mice on day -1 and + 4, intravenously (i.v.), at a dose of 600 mug of protein in 0.2 ml sterile vehicle. One ml of rabbit anti-rmGM-CSF antibody is able to neutralize a minimum of 400,000 units of rmGM-CSF, as attested by supplier. Control mice received equivalent amounts of the IgG fraction of normal rabbit serum.

Target cells

PE, the target cells, were prepared as reported [31]. Briefly, blood from mice having 60-80% parasitaemia was collected in acid-citrate-dextrose saline, spun at 450 g for 10 min at 4° C, and the PE-rich layer was aspirated, aseptically. The PE were washed (x 3) with sterile Hank's balanced salt solution (HBSS), resuspended (1×10^7/ml) in culture medium, and opsonized by incubating (37° C; 30 min) with diluted serum (1:15 in HBSS) from mice refractory to repeated massive *P. berghei* challenges.

Total leukocyte count

Tail blood samples from control and treated mice were diluted in Turk's fluid. The number of leukocytes/mul blood was counted using a Nuber-Levy-Hausser chamber.

Preparation of adherent peritoneal and splenic macrophages

Two ml of peritoneal exudate cell (PEC) suspension, collected by injecting (i.p.) 5.0 ml of chilled HBSS and lavage, from each mouse, was centrifuged (500 g; 7 min; 4° C). The cell pellets were resuspended in 5.0 ml Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2.0 mM L-glutamine, 0.01 M HEPES, 5×10^{-5} M 2-mercaptoethanol and 40 mug/ml gentamicin (CDMEM). The adherent macrophages were harvested, resuspended in 5.0 ml HBSS and counted (number/ml). Similarly, mouse splenic macrophages were harvested by finely mincing the aseptically removed spleens in plastic dishes containing 5.0 ml of DMEM. After allowing the large pieces to settle for 5 min at room temperature, the cell suspension was centrifuged (500 g; 7 min; 4° C). The cell pellet was then resuspended in 5.0 ml of CDMEM, and the adherent macrophages were harvested and counted as for peritoneal macrophages. Separate sets of experiments were run for pe-ritoneal and splenic macrophages for day +7 and day +16. The DMEM and HBSS contained < 0.1 ng/ml

endotoxin as determined by chromogenic *Limulus* amoebocyte lysate test (Sigma). Macrophages were > 96% pure according to morphological, phagocytic and non-esterase staining criteria, and > 98% viable as judged by trypan blue exclusion.

Determination of the phagocytic activity of peritoneal and splenic macrophages

Phagocytic activity was determined as described [33]. Briefly, 100 mul of peritoneal or splenic macrophage suspension (1×10^7 cells/ml) from co-administered or control mice were layered over 12 mm, round cover slips, and incubated at 37° C for 1 hour in humid 5% CO_2-air atmosphere to allow the formation of macrophage monolayer. The macrophages were washed with DMEM at 37° C, and then overlaid with 1×10^7 opsonized PE in CDMEM. Following incubation at 37° C for 30 min, the assay was stopped by adding an excess of ice-cold medium, the macrophages were washed with phosphate-buffered saline (pH 7.2) diluted with distilled water (1:5), and stained with Giemsa to assess the ingestion. Two hundred macrophages on each cover slip were examined by light microscopy, and the number of PE ingested/100 macrophages was determined. All experiments were run in triplicate, separately. Student's *t*-test was used for statistical analysis and $p < 0.05$ was considered significant.

RESULTS

Effect of rmGM-CSF and M-ENK administration on the course of P. berghei *infection in mice*

Co-administration of 10.0 mug/kg rmGM-CSF and 2.0 mg/kg M-ENK x 3/day in *P. berghei*-infected mice, from day -1 through day +4, resulted in significant suppression ($p < 0.05$) (in some cases even complete elimination) of parasitaemia, compared to vehicle-treated controls (Figure 1). The mice (n = 6) became positive on day +5, developed maximum (20.4 ± 3.8%) parasitaemia on day +15, and gradually turned negative by day +19. Whereas four of these mice completely eliminated the parasites (as determined by isodiagnostic tests) and remained negative till the end of experiment on day +60, the other 2 mice became positive on day +24, developed > 65.0% parasitaemia on day +31, and died (data not shown). Somewhat similarly, mice co-administered with 5.0 mug/kg rmGM-CSF and 2.0 mg/kg M-ENK x 3/day, from day -1 through to day +4,

showed significant ($p < 0.05$) suppression of parasitaemia until day +18, which then increased progressively (66.5 ± 10.2%), and finally culminated in the death of all the mice by day +21. The course of parasitaemia in mice co-administered with 1.25 or 0.312 mug/kg rmGM-CSF and 2.0 mg/kg M-ENK x 3/day, from day -1 to day +4, was almost similar to that in the vehicle-treated controls. Surprisingly, co-administration of 0.312, 1.25, 5.0 and 10.0 mug/kg rmGM-CSF and 10.0 mg/kg M-ENK x 3/day in mice, from day -1 through day +4, also appeared to lack any effect in the course of infection, as compared to controls (data not shown). Furthermore, mice that received either rmGM-CSF (0.312, 1.25, 5.0 and 10.0 mug/kg x 3/day) or M-ENK (2.0 and 10.0 mg/kg x 3/day), alone, from day -1 to day +4, showed no detectable change in the course of parasitaemia, as compared to the controls (data not shown). Curiously, both rmGM-CSF and M-ENK, separately or combined, lacked any direct antimalarial activity against *P. berghei* and *P. falciparum*, *in vitro* (data not shown).

Effect of anti-rmGM-CSF polyclonal antibody treatment on the rmGM-CSF and M-ENK co-administration-induced suppression of parasitaemia in P. berghei *-infected mice*

Simultaneous treatment of *P. berghei*-infected mice with rabbit anti-rmGM-CSF polyclonal antibody at a dose of 600 mug on day -1 and + 4, and 10.0 mug/kg rmGM-CSF and 2.0 mg/kg M-ENK x 3/day, from day -1 through to day +4, abrogated the suppression of parasitaemia; the course of infection in these mice was similar to that in the vehicle-treated controls (Figure 2). Paradoxically, out of the 6 mice that were given similar treatment but with pre-immune rabbit IgG at a dose of 600 mug, 4 mice were protected; the remaining 2 succumbed to infection.

Effect of naloxone treatment on the course of P. berghei *infection in mice co-administered with rmGM-CSF and M-ENK*

Naloxone treatment (10.0 mg/kg/day; i.p.; day -1 through day +4) of *P. berghei*-infected mice co-administered with protective doses of rmGM-CSF and M-ENK completely inhibited the suppression of parasitaemia (Figure 3).

Effect of rmGM-CSF and M-ENK co-administration on the number of circulating leukocytes, and the pool-size and phagocytic activity of peritoneal and splenic macrophages in P. berghei *-infected mice*

Total leukocyte counts of *P. berghei*-infected mice co-administered with rmGM-CSF and M-ENK were performed on day +7 and day +16. The cell counts on day +7 remained unchanged (Table 1); however, there was almost a 2-fold increase in the number of neutrophils. On the other hand, the day +16 cell counts of protected mice showed a clear leukocytosis, and there were almost 3- and 5-fold increases in neutrophils and monocytes, respectively. Figure 4 shows pooled data on peritoneal macrophages from three separate experiments wherein *P. berghei*-infected mice were co-administered with 10.0 mug/kg rmGM-CSF and 2.0 mg/kg M-ENK x 3/day, from day -1 through day +4. A clear increase in the number of peritoneal macrophages was observed both on day +7 (almost 6-fold) and on day +16 (almost 8.8-fold). Similarly, in spleen an almost 2.8-fold increase in the number of macrophages was observed on day +7, along with a moderate rise (30%) in the spleen weight (data not shown); however, on day +16, whereas there was seemingly no further change in the macrophage populations, a heavy splenomegaly was evident with nearly 50-58% increase in weight (data not shown). The percentage of phagocytic peritoneal macrophages from these co-administered mice showed a significant ($p < 0.05$) increase on day +7 (nearly 5.5-fold) over the serum/saline- or untreated controls, whereas on day +16, close to a 8.7-fold enhancement in phagocytosis was observed (Figure 5). Similarly, the percentage of splenic macrophages engaged in phagocytosis showed an increase of up to 3- and 3.5-fold on day +7 and +16, respectively. Besides the increase in the pool-size and percentage of macrophages engaged in phagocytosis, there was a clear-cut augmentation in the intrinsic phagocytic activity of macrophages as demonstrated by the increase in the average number of PE/phagocytically active macrophages; peritoneal macrophages: 6- and 8-fold, and splenic macrophages: 5.5- and 6-fold on day +7 and +16, respectively (Figure 6).

Effect of silica on the course of P. berghei *infection in mice co-administered with rmGM-CSF and M-ENK*

A single administration of sterile silica (3.0 mg/mouse; i.v.), on day 0, in *P. berghei*-infected mice co-administered with protective doses of rmGM-CSF and M-ENK, almost completely abrogated their combined protective effect (Figure 7).

DISCUSSION

Our laboratory is engaged in research into the biotherapy of malaria. In this study, we have attempted to determine the protective effect of rmGM-CSF and M-ENK co-administration on the course of *P. berghei* infection in mice. Our results clearly demonstrate that co-administration of rmGM-CSF and dose dependent quantities of M-ENK in *P. berghei*-infected mice can strongly suppress (in some cases even completely eliminate) the parasitaemia; however, when administered alone, none of these agents could induce detectable protection. Silica, a selective killer of macrophages [34], abrogated the combined protective effect of both of these agents.

Metcalf *et al.* [35] have reported that in BALB/c mice given 65 ng rmGM-CSF, i.p., the serum GM-CSF level attained a peak value of 500 U/ml after 30 min and then, by 3 hours, fell logarithmically to 10 U/ml (approximate half-life = 35 min), a concentration that would have minimal effect on phagocytes and hematopoiesis. Based on these considerations, they chose a dose range of 6 ng to 200 ng/mouse, 3 times/day for 6 days, in their studies. The interrupted schedule of 200 ng doses, thus would have achieved > 50 U/ml (a concentration considered significant from *in vitro* studies) for up to 3 hours, followed by an interlude of 5-7 hours with no significant elevation of CSF levels. Therefore, in the present studies, we considered it appropriate to use a similar range of rmGM-CSF doses; the range of serum concentrations achieved by these doses were within those estimated in the serum during the cytokine-cascade due to natural parasitic infections [36]. The doses of M-ENK were also selected based on the published reports [37], wherein both immunoenhancing and immunosuppressive effects of M-ENK were reported in mice following i.p. injections of multiple doses of 2.0 and 10.0 mg/kg/day x 4-8 days, respectively. Furthermore, in mice chronically treated with enkephalins, these authors observed no obvious behavioral signs which were different from the animals' normal behaviour in the laboratory.

In this study, *P. berghei*-infected mice were adminis-tered with rmGM-CSF (0.312, 1.25, 5.0 and 10.0 mug/kg x 3/day) and/or M-ENK (2.0 and 10.0 mg/kg x 3/day), from day -1 to day +4. Whereas the effect of rmGM-CSF was predominantly dose-dependent, the effect of M-ENK appeared to be both dose-dependent and biphasic, within the dose limits tested, as regards both the course of infection, and in a contemporaneous manner, the alterations in the numerical strength and the phagocytic activity of the macrophages. Unfortunately however, no generally accepted satisfactory explanation can be

advanced for these latter observations. Nevertheless, these observations are consistent with similar activities of endorphins [38], morphine [31, 32] and enkephalins [39, 40] all of which have a characteristic bell-shaped, dose-response curve, which indicate the mediation by multiple opioid receptors which transduce bi-directional paradoxical signals which culminate in the translation of opposing biological effects. Besides this however, other alternative explanations [41, 42] also can not be ruled out.

In quantitative terms, the single most striking change, in co-administered and protected mice, was a dramatic alteration in the number of leukocytes both locally (in the peritoneal cavity; the site of injection) and systemically (in the circulation and spleen). There was an almost 15-fold rise in the number of peritoneal macrophages (with an approximately 35-fold increase in the mitotic activity; data not shown). The peritoneal macrophages from co-administered and protected mice, on day +16, exhibited rises both in the percentage of macrophages with phagocytosed PE (8.7-fold) and in the average number of IE phagocytosed per macrophage (8-fold). These data, in combination with the absolute increase in the total number of macrophages induced by rmGM-CSF and M-ENK, especially in the peritoneal cavity, indicated that the over-all level of phagocytic activity in the total peritoneal cavity macrophage population had increased > 100-fold, relative to the control mice given serum/saline. These observations, therefore, confirm the earlier reports demonstrating that i.p. administration of GM-CSF to mice and humans elicits large increases in the number and functional capacity of peritoneal macrophages [6, 35, 43, 44]. Since macrophage phagocytosis of PE is known to play a major role in the expression of protective immunity in malaria by clearing and destroying the parasites from the circulation [45], and since recovery from human malaria correlates with the phagocytosis of IE by mononuclear cells [46], it is very likely that this may be one of the effector immune mechanism, among others that may be responsible for imparting protection in our studies. Systemically, the rmGM-CSF and M-ENK co-administration in *P. berghei*-infected mice induced only a 2-fold increase in the number of circulating neutrophils; the level of eosinophils and monocytes remained almost unaltered. The spleens of co-administered and protected mice, on day +16, showed a moderate dose-dependent rise in weight nearing 38%, which was very closely paralleled by corresponding increases in the cellularity (data not shown). The differential cell counts revealed a consistent 2.6-fold increase in the percentage of macrophages. Surprisingly, the percentage of neutrophils and eosinophils remained unchanged. *Ex vivo*, the percentage

increase in splenic phagocytic cells on day +7 and day +16 was observed to be 3- and 7-fold, respectively. Similarly, the average number of PE/splenic macrophage also rose by 4- and 8-fold on day +7 and +16, respectively.

GM-CSF is known to act on various immunocompetent cell types including macrophages [47] through specific cell surface receptors. rmGM-CSF has been reported to activate macrophages to kill *Leishmania tropica* and *L. donovani* [7, 10], to inhibit the growth and multiplication of *Trypanosoma cruzi* and for the release of hydrogen peroxide [9]. Furthermore, rmGM-CSF is known to function as an intermediate in the increased production of tumor necrosis factor, and macrophage accumulation in the lymphoid organs during murine cerebral malaria [48]. GM-CSF is also known to increase the number of splenocytes capable of secreting interferon-gamma and interleukin-2, but its effect on antibody production is complex and elusive [22]. M-ENK, on the other hand, has been shown to have significant effects on the immune system *via* pharmacological interactions with opioid-receptors on the surface of immunocytes, and is known to enhance several pro-inflammatory, host defence functions such as macrophage phagocytosis [39, 49], modulation of the elaboration of phagocytosis promoting lymphokines by the splenocytes [30], and the production of CSFs by macrophages stimulated with plasmodial antigens [32], *in vitro*. Nevertheless, based on the evidence such as (i) both the number and phagocytic/microbicidal activity of macrophages are augmented by GM-CSF, (ii) macrophages function as the key cellular target for the expression of the immunomodulatory effects of opioids, (iii) macrophages play a definitive role in the resolution of malaria infection, and (iv) opioids can modulate the plasmodial antigen-induced production of CSFs by macrophages, we hypothesize that rmGM-CSF and M-ENK-induced protection against malaria may be due to the macrophage effector mechanisms. However, other direct or indirect protective mechanism(s) may also be involved.

What then is the biological significance of these studies? The potential toxicological effects of recombinant human GM-CSF (rhGM-CSF) are now well known [50-53], and it is used clinically to stimulate the growth of bone marrow cells in several different settings [52]. rhGM-CSF has also been known to enhance the antibody response to recombinant hepatitis B vaccine [53], and human GM-CSF has been tested as an adjuvant in the immunotherapy of human cancer [54]. M-ENK has also been clinically tested in AIDS and cancer patients with several of their immune parameters responding positively [28]. We have

observed no ill effects in mice co-administered with rmGM-CSF and M-ENK. Because recovery from malaria in Gambian children infected with *P. falciparum* has been correlated with the phagocytosis of PE by macrophages, and because in our studies too the observed protection against malaria in rmGM-CSF and M-ENK co-administered mice appeared to strongly correlate with the macrophage-mediated protective mechanisms, the results of this study can be objectively extrapolated to humans, albeit after expedient extended studies in non-human primate malaria models which should demonstrate acceptable levels of protection. Furthermore, these studies should be extended to sporozoite-induced malarias, as both GM-CSF and M-ENK can be expected to inhibit the growth and multiplication of different liver stages of the malaria parasite.

CONCLUSION

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Figures

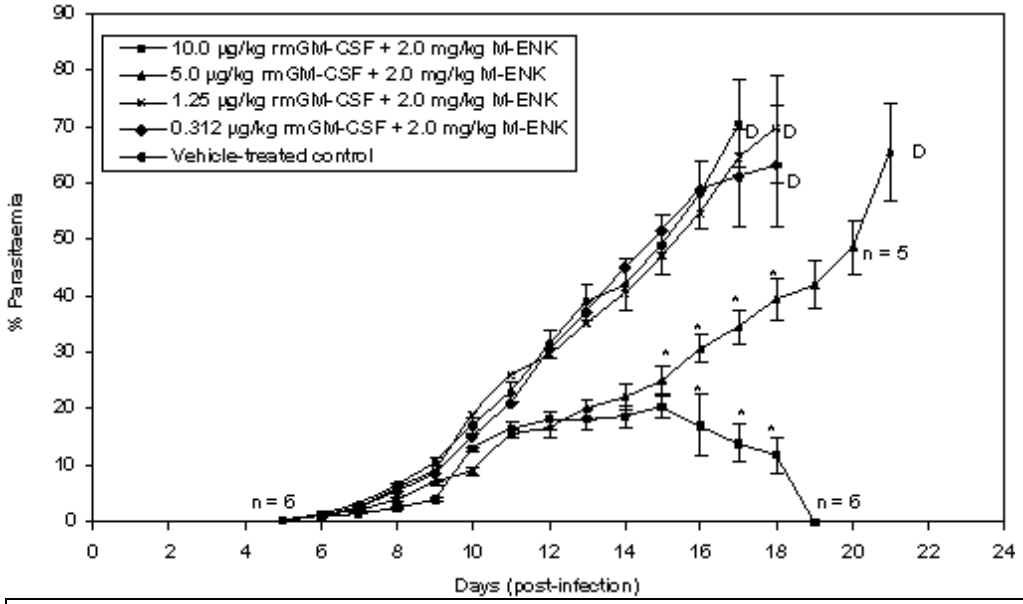


Figure 1

Course of *P. berghei* infection in mice co-administered with rmGM-CSF and M-ENK.

Mice (6/group) were co-administered with various doses of rmGM-CSF (as indicated in the in-set) and 2.0 mg/kg M-ENK x 3/day, in 0.2 ml sterile 5.0% normal mouse serum/saline (vehicle), i.p., from day -1 through day +4. Controls received vehicle only. For infection, mice were inoculated with 1 x 10^3 PE, i.p., on day 0. Parasitaemia has been expressed as percentage PE ± S.D. n, number of mice. D, died. * Significantly different ($p < 0.05$) from control group.

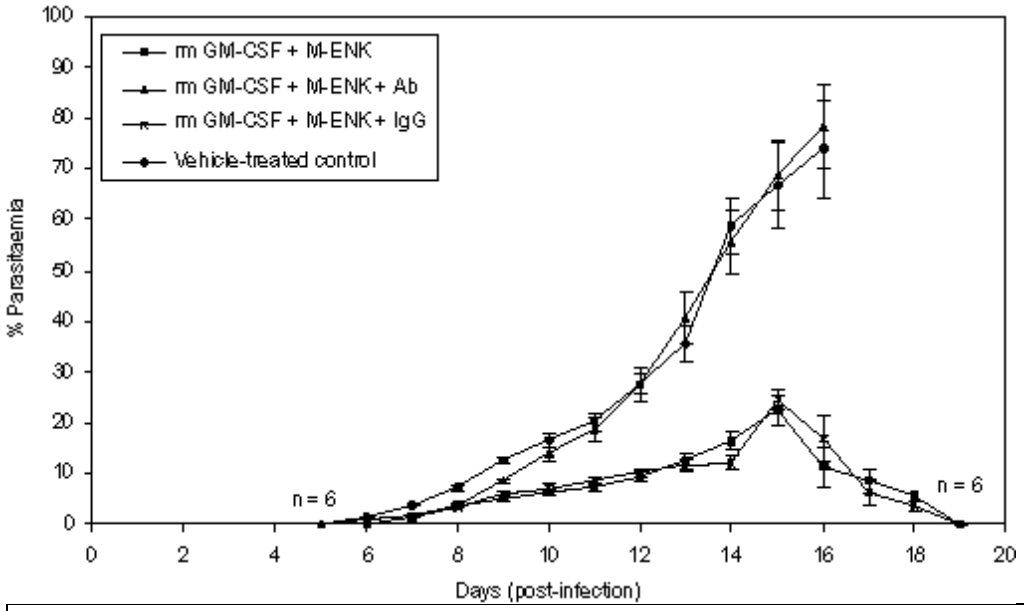


Figure 2

Effect of rabbit anti-rmGM-CSF polyclonal antibody on the course of *P. berghei* infection in mice co-administered with rmGM-CSF (10.0 mug/kg) and M-ENK (2.0 mg/kg) x 3/day, i.p., from day -1 through day +4.

Mice (6/group) were simultaneously treated with antibody (600 mug of protein in 0.2 ml vehicle) on day -1 and day +4, i.v. Controls received equivalent amounts of normal rabbit IgG in vehicle. Other details as for Figure 1.

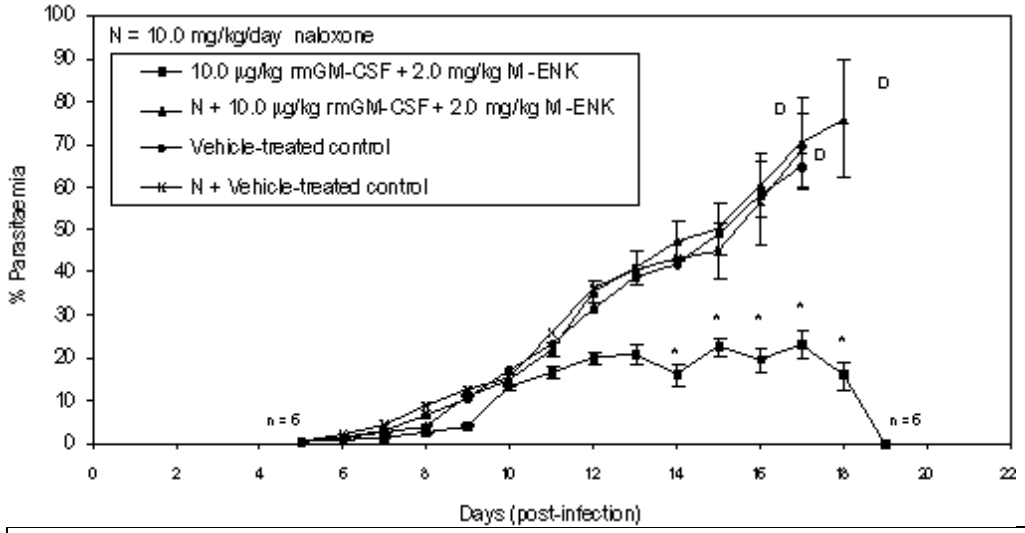


Figure 3
Effect of naloxone treatment on the course of *P. berghei* infection in mice co-administered with rmGM-CSF (10.0 mug/kg) and M-ENK (2.0 mg/kg) x 3/day, i.p., from day -1 through day +4.

Mice (6/group) were treated with naloxone on day -1 through day +4, i.p. Controls received vehicle only. Other details as for Figure 1.

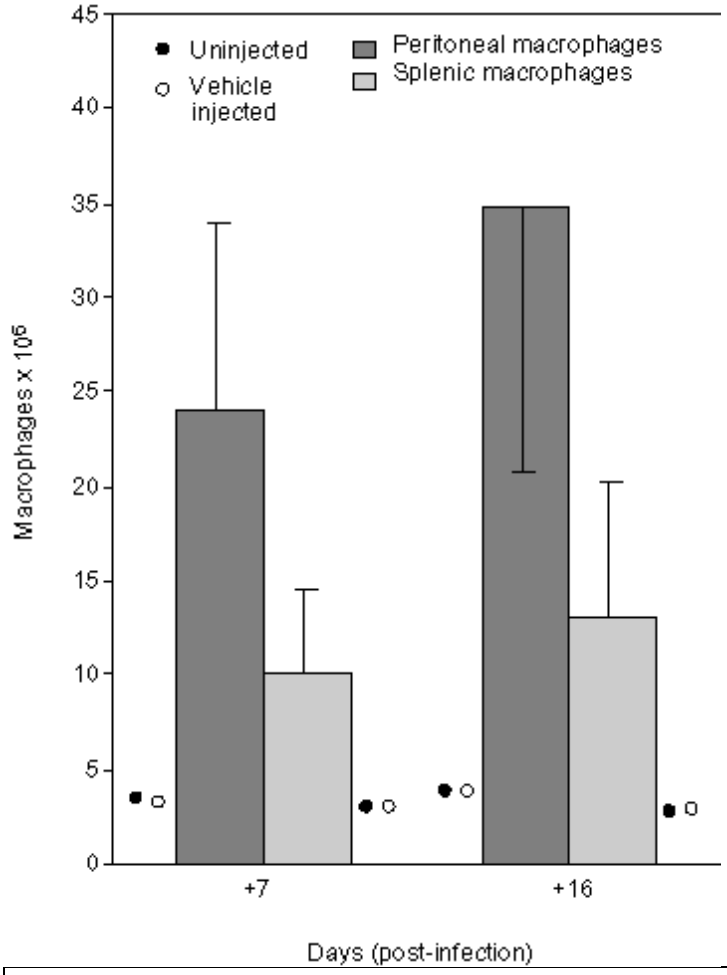


Figure 4
Changes in the number of macrophages in the peritoneal cavity and spleen in *P. berghei*-infected mice co-administered with rmGM-CSF (10.0 mug/kg) and M-ENK (2.0 mg/kg) x 3/day, i.p., from day -1 through day +4.

Values are mean ± S.D. from 6 mice. The numbers of macrophages in both peritoneal cavity and spleen on day +7 and day +16 were significantly ($p < 0.05$) higher than in the vehicle-injected control mice. Other details as for Figure 1.

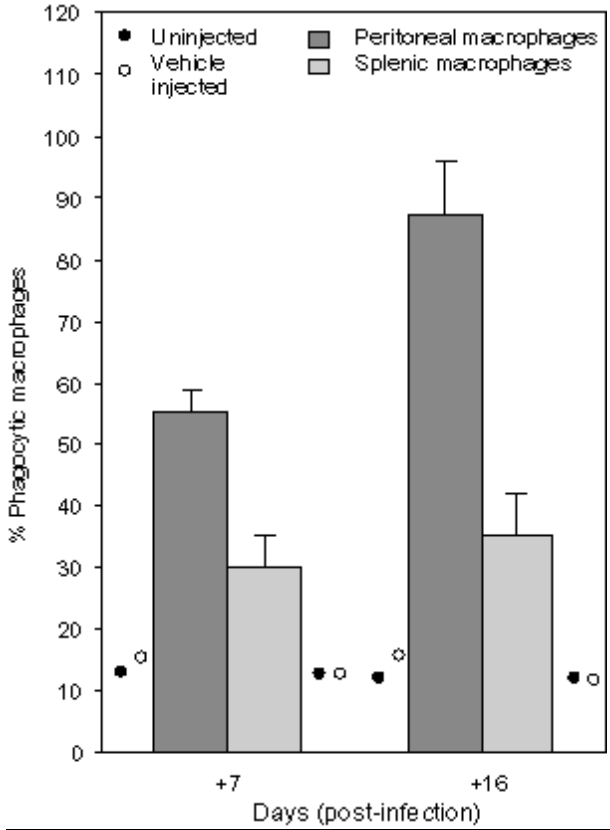


Figure 5

Ex vivo **percentage of phagocytic macrophages from** ***P. berghei*****-infected mice co-administered with rmGM-CSF (10.0** mu**g/kg) and M-ENK (2.0 mg/kg)** x **3/day, i.p., from day -1 through day +4.**

Opsonized PE were used as the target cells. Values are mean ± S.D. of three experiments run in triplicate, separately. Other details as for Figure 1.

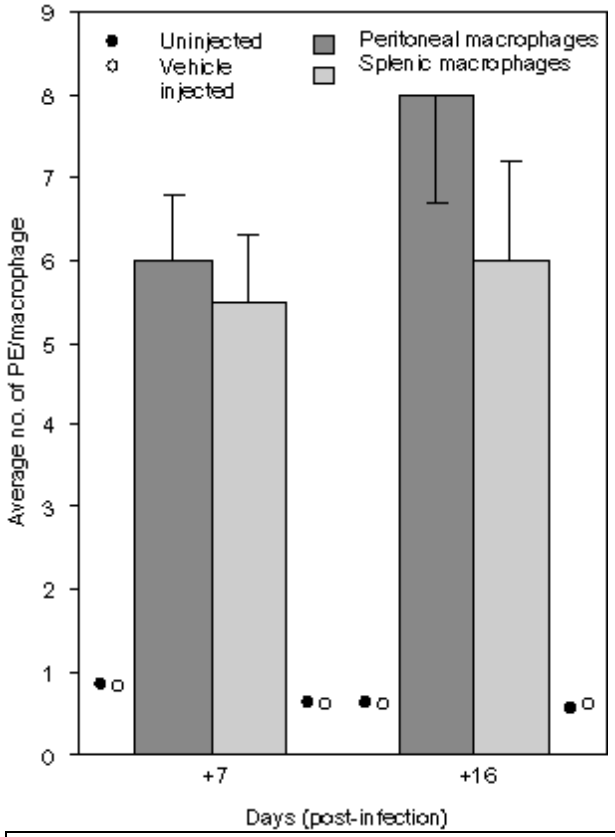


Figure 6

Average number of opsonized PE/macrophage from *P. berghei*-infected mice co-administered with rmGM-CSF (10.0 mug/kg) and M-ENK (2.0 mg/kg) x 3/day, i.p., from day -1 through day +4. Details as for Figures 1 and 5.

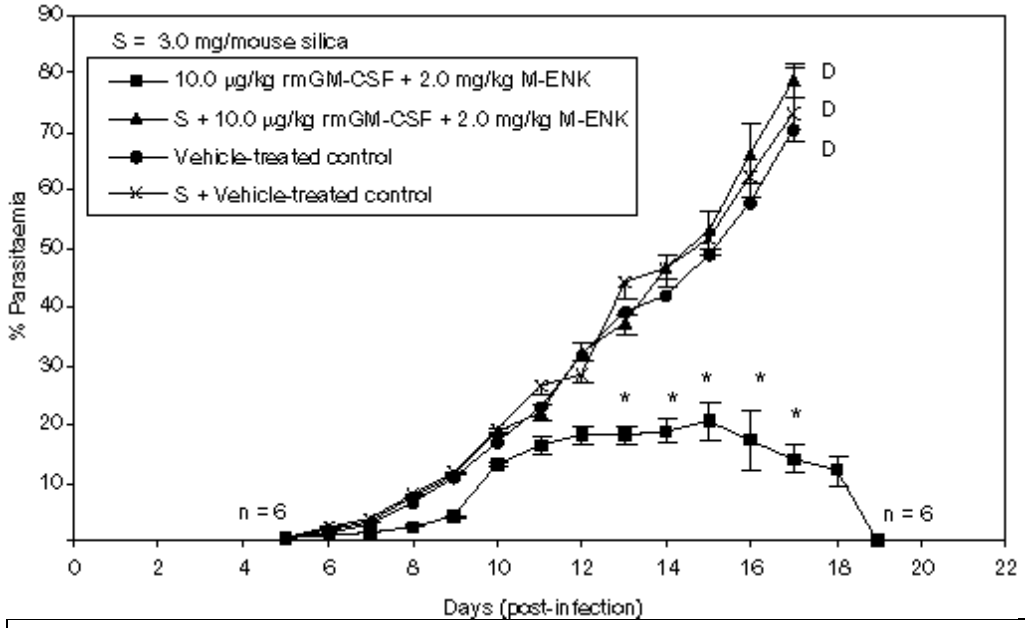


Figure 7

Effect of silica treatment on the course of *P. berghei* infection in mice co-administered with rmGM-CSF (10.0 mug/kg) and M-ENK (2.0 mg/kg) x 3/day, i.p., from day -1 through day +4.

Mice (6/group) were treated with silica (particle size < 5.0 mu; sterilized by hot-air overnight) in 0.2 ml sterile vehicle, i.v., 15 min after the initiation of infection. Controls received vehicle only. Other details as for Figure 1.

Table 1
Effect of rmGM-CSF and M-ENK co-administration on leukocyte counts in *P. berghei*-infected mice[a]

Treatment	Day	Total leukocytes/ mul blood[b]	Total cells/mul blood[b]			
			Neutrophils	Lymphocytes	Eosinophils	Monocytes
–	7	9,501 ± 3,600	1,400 ± 600	7,100 ± 315	50 ± 450	550 ± 210
–	16	–	–	–	–	–
Serum/saline	7	8,600 ± 3,750	1,640 ± 660	6,700 ± 2, 320	80 ± 60	470 ± 220
	16	–	–	–	–	–
rm GM-CSF	7	10,960 ± 281	3,600 ± 8,500	6,500 ± 1,400	85 ± 65	450 ± 200
(10.0 mug/kg)	16	–	–	–	–	–
M-ENK	7	9,200 ± 3,700	1,500 ± 700	7,200 ± 2,900	50 ± 40	680 ± 310
(2.0 mg/kg)	16	–	–	–	–	–
M-ENK	7	6,700 ± 1,400	1,140 ± 200	4,000 ± 2,200	55 ± 40	340 ± 110
(10.0 mg/kg)	16	–	–	–	–	–
rm GM-CSF	7	10,940 ± 3,550	3,200 ± 800*	6,900 ± 2,320	80 ± 60	660 ± 270
(10.0 mug/kg)		13,300 ± 4,500	3,900 ± 950*	7,700 ± 3,350	85 ± 65	2,750 ± 830*
+ M-ENK	16					
(2.0 mg/kg)						
rm GM-CSF	7	10,500 ± 2,780	3,500 ± 750*	7,100 ± 2,700	75 ± 45	590 ± 240
(10.0 mug/kg)	16	13,600 ± 3,800	4,100 ± 700*	7,400 ± 3,800	80 ± 55	780 ± 310
+ M-ENK						
(10.0 mg/kg)						

[a] Mice (6/group) were co-administered with various doses of rmGM-CSF and M-ENK × 3/day, in 0.2 ml sterile 5.0% normal mouse serum/saline (vehicle), i.p., from day –1 through day +4. Controls received vehicle only. For infection, mice were inoculated with 1×10^3 PE, i.p., on day 0.
[b] Data are mean ± S.D. of three separate determinations from each of six mice.
* Values are significantly different ($p < 0.05$) from control group.

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In this issue

Virus-cell interactions: impact on cytokine production, immune evasion and tumor growth

T. H. Mogensen, S. R. Paludan
virus, cytokines, signal transduction, immune evasion, tumor growth.

Safety, pharmacokinetics and pharmacodynamics of recombinant human tumour necrosis factor-binding protein-1 (Onercept) injected by intravenous, intramuscular and subcutaneous routes into healthy volunteers

I. Trinchard-Lugan, Q. Ho-Nguyen, W.M. Bilham, M. Buraglio, A. Ythier, A. Munafo
recombinant human tumour necrosis factor-binding protein-1 (r-hTBP-1), safety, pharmacokinetics, pharmacodynamics.

Direct effects of tumor necrosis factor alpha (TNF-alpha) on murine skeletal muscle cell lines. Bimodal effects on protein metabolism

B. Alvarez, L. S. Quinn, S. Busquets, F. J. López-Soriano, J. M. Argilés
cytokines, myotubes, cachexia, protein turnover.

Receptor-like properties of the 26 kDa transmembrane form of TNF

A. Domonkos, A. Udvardy, L. László, T. Nagy, E. Duda
cytokine, TNF family, receptor, signaling, reverse-signaling.

Local administration of interleukin-1beta for the treatment of lung abscesses induces neutrophil activation and changes in proinflammation cytokine production

A. Simbirtsev, E. Variouchina, V. Konusova, A. Kotov, S. Ketlinsky, A. Salamatov, L. Bisenkov
recombinant human interleukin-1beta, lung inflammation, abscess, neutrophils, cytokines.

Effects of interleukin-2 therapy on the proliferation and differentiation of CD4/CD25 positive and CD4/CD25 negative cells in HIV+ patients

L. Caggiari, S. Zanussi, M. D'Andrea, M. T. Bortolin, C. Crepaldi, C. Caffau, P. de Paoli
cytokines, Ki67, intracellular IFN-gamma.

Tumor necrosis factor-alpha and nitric oxide in vertically HIV-1-infected children: implications for pathogenesis

J. González-Nicolás, S. Resino, J. L. Jiménez, S. Alvarez, M. Fresno, M. A. Muñoz-Fernández
tumor necrosis factor, nitric oxide, HIV-infection, immunological and virological markers.

Serum levels of angiogenic cytokines in systemic lupus erythematosus and their correlation with disease activity

E. Robak, A. Wozniacka, A. Sysa-Jedrzejowska, H. Stepien, T. Robak
systemic lupus erythematosus, disease activity, angiogenesis, platelets, hepatocyte growth factor, vascular endothelial growth factor, basic fibroblast growth factor, transforming growth factor beta.

Modulation of the allergen-induced human IgE response in Hu-SCID mice: inhibitory effect of human recombinant IFN-gamma and allergen-derived lipopeptide

C. Duez, H. Gras-Masse, H. Hammad, H. Akoum, A. Didierlaurent, C. André, A.-B. Tonnel, J. Pestel
SCID mouse, IgE, IFN-gamma, lipopeptide, house dust mite.

Cytokine-inducing activity of a proline-rich polypeptide complex (PRP) from ovine colostrum and its active nonapeptide fragment analogs

A. Zablocka, M. Janusz, K. Rybka, I. Wirkus-Romanowska, G. Kupryszewski, J. Lisowski
ovine colostrum, proline-rich polypeptide complex (PRP), nonapeptide (NP) and hexapeptide (HP) from PRP, oligomers of NP and HP, cytokines.

Quantitation of chemokines (MDC, TARC) expression in mucosa from Crohn's disease and ulcerative colitis

F. Jugde, M. Alizadeh, C. Boissier, D. Chantry, L. Siproudhis, S. Corbinais, E. Quelvennec, F. Dyard, J.-P. Campion, M. Gosselin, J.-F. Bretagne, G. Sémana, D. Heresbach
real-time TR-PCR, chemokines, Crohn’s disease, ulcerative colitis.

Activation of caspase-3 by interferon alpha causes interleukin-16 secretion but fails to modulate activation induced cell death

O. Ludwiczek, A. Kaser, R. O. Koch, W. Vogel, W. W. Cruikshank, H. Tilg
interleukin-16, interferon-alpha, caspase-3.

Cytokine deviation induced by intrathymic injection of staphylococcal enterotoxin B (SEB)

P. Goettelfinger, F. Lecerf, S. Berrih-Aknin, M. German-Fattal
SEB, intrathymic injection, anergy, cytokine deviation, quantitative RT-PCR.

Strain-dependent difference in inducible nitric oxide synthesis (iNOS) expression in rat pancreatic islets correlates with interferon regulating factor 1 (IRF-1) and heat shock protein 70 (HSP70) expression

J. Johannesen, F. Pociot, A. E. Karlsen, T. Mandrup-Poulsen, J. Nerup
type 1 diabetes, islets of Langerhans, beta-cells, cytokines, interleukin-1beta, interleukin-1beta receptor type I, nitric oxide, inducible nitric oxide synthase, heat shock protein 70, interferon regulating factor 1.

Neutrophil apoptosis in autoimmune Fas-defective MRL lpr/lpr mice

E. Del Giudice, A. Ciaramella, N. Balestro, D. Neumann, P. G. Romano, M. P. Cesaroni, G. Maurizi, P. Ruggiero, D. Boraschi, P. Bossù
neutrophils, apoptosis, autoimmunity.

A role for interferon-gamma in the hypermetabolic response to murine toxoplasmosis

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toxoplasma, hypermetabolism, reactive oxygen species, lipid peroxidation, UCP1, interferon-gamma.

Protection of mice from malaria after co-administration of recombinant mouse granulocyte-macrophage colony-stimulating factor and methionine-enkephalin

P. Pal Singh, S. Singh
Plasmodium berghei, protection, rmGM-CSF, met-enkephalin.

Semiautomated and simultaneous analysis of the interleukin-10 gene microsatellites IL-10G and IL-10R by fluorescence-based polymerase chain reaction reveals significant differences in allele distributions between Caucasians (Germany) and Africans (Gabon)

D. Kube, D. Schmidt, M. Mörmann, A.C. Uhlemann, J. Tomiuk, H. Tesch, P.G. Kremsner
microsatellites, interleukin-10 promoter, multiplex-PCR, Caucasian, African.

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