Treatment with recombinant human tumour necrosis factor-α reduces parasitaemia and prevents *Plasmodium berghei* K173-induced experimental cerebral malaria in mice

N. S. POSTMA[1]*, C. C. HERMSEN[2], D. J. A. CROMMELIN[1], J. ZUIDEMA[1] *and* W. M. C. ELING[2]

[1] *Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands*
[2] *Department of Medical Microbiology, University Hospital Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands*

(*Received 29 April 1998; revised 21 July 1998; accepted 21 July 1998*)

SUMMARY

The present study shows that treatment with recombinant human tumour necrosis factor-α (rhTNF-α) can suppress parasitaemia and prevents development of experimental cerebral malaria (ECM) in *Plasmodium berghei* K173-infected mice. Mice received rhTNF-α treatment either by subcutaneous injection of free or liposome-encapsulated rhTNF-α or sustained intraperitoneal administration of rhTNF-α given via mini-osmotic pumps. Low-dose treatment with a subcutaneous bolus injection of rhTNF-α protected against ECM when treatment was started on day 5 or 6 after infection. The same protective efficacy was obtained either by subcutaneous injection of liposome-encapsulated rhTNF-α or by sustained release from osmotic pumps, but in the latter case a 10-fold lower daily dose of rhTNF-α was sufficient. Treatment with rhTNF-α substantially suppressed parasitaemia in ECM-protected mice, but not in mice developing ECM. Thus, the rhTNF-α mediated suppression of parasitaemia is directly or indirectly involved in protection against ECM. Sustained delivery of rhTNF-α through osmotic pumps, but not by subcutaneous injection of liposome-encapsulated rhTNF-α, resulted in increased concentrations of soluble mouse TNF receptor R75 (sTNFR75) in plasma at day 9 after infection when non-treated mice die of ECM. Thus, protection against ECM is not directly correlated with the sTNFR75 concentrations at day 9 after infection.

Key words: *Plasmodium berghei* K173, experimental cerebral malaria, recombinant human tumour necrosis factor-α, liposomes, continuous infusion, TNF receptors.

INTRODUCTION

Tumour necrosis factor-α (TNF-α) plays a dual role in experimental murine malaria. As anticipated by Clark (1978), several studies now provide evidence that TNF-α suppresses the parasite, e.g. *Plasmodium yoelii* (Taverne *et al*. 1994) and *P. chabaudi AS* (Jacobs, Radzich & Stevenson, 1996), while TNF-α also contributes to the development of experimental cerebral malaria (ECM) in *P. berghei* ANKA-infected mice (reviewed by Grau *et al*. 1989). In human *P. falciparum* malaria, high concentrations of immunoreactive TNF-α have been associated with both disease severity and cerebral malaria (Kwiatkowski *et al*. 1990). Curfs *et al*. (1993) demonstrated that administration of high doses of recombinant human TNF-α (rhTNF-α) to *P. berghei* K173-infected mice induced pathology in the brains that resembled the pathology found in the brains of non-treated mice dying of ECM, while low-dose rhTNF-α treatment protected against ECM. The latter observation merits further attention.

A possible mechanism involved in the rhTNF-α mediated protection against ECM is the production of soluble TNF receptors (sTNFRs) (Engelmann, Novick & Wallach, 1990). Soluble TNFRs are derived from proteolytic cleavage of the cellular receptors TNFR55 and TNFR75. They competitively inhibit interaction of TNF-α with the membrane-bound receptor (Lesslauer *et al*. 1991) and thus provide a possible mechanism for regulating the availability of TNF-α. In another observation, repeated administration of rhTNF-α resulted in the development of tolerance to the lethal effects of TNF-α (Takahashi, Brouckaert & Fiers, 1995). Sustained release (from osmotic pumps or liposomes) of small amounts of rhTNF-α might prevent development of the cerebral syndrome e.g. through induction of refractoriness of TNF-α sensitive cells

* Corresponding author: Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands. Tel: 31 30 2537304. Fax: +31 30 2517839. E-mail: n.s.postma@pharm.uu.nl

https://doi.org/10.1017/S0031182098003552 Published online by Cambridge University Press

to subsequent stimuli (Alexander *et al*. 1991). The successful use of liposomes as carrier systems for sustained release of encapsulated agents in general (Kadir, Zuidema & Crommelin, 1993) and in the treatment of malaria (Peeters *et al*. 1989) has been described before.

The aim of the present study was to analyse the effect of treatment with low doses of rhTNF-α on the protection against the development of ECM in *P. berghei* K173-infected mice.

MATERIALS AND METHODS

Materials

Highly purified rhTNF-α from *Escherichia coli* (Wang *et al*. 1985) with a biological activity of $2{\cdot}5 \times 10^7$ U/mg (Creasey *et al*. 1987) was a kind gift of Chiron Corporation (Emeryville, California USA). Egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG) were donated by Lipoid (Ludwigshafen, Germany). All other reagents used were of analytical grade.

Preparation of liposomes

A mixture of EPC and EPG (9:1, molar ratio) was dissolved in ethanol and evaporated to dryness in a rotary evaporator under reduced pressure at 35 °C. After drying the lipid film with nitrogen for at least 20 min, the lipid film was hydrated with the rhTNF-α solution (100 μg rhTNF-α/ml in 20 mM Hepes, 149 mM NaCl, pH 7·4, at a lipid concentration of 40 mM). The resulting liposome dispersion (MLVs) was centrifuged 3 times ($2{\cdot}7 \times 10^5$ ***g*** for 30–60 min at 4 °C) to remove the non-liposomal rhTNF-α (Beckman Optima™ LE-80K, Beckman Instruments, Palo Alto, CA).

Empty or buffer-loaded liposomes were prepared in the same way, but hydrated with Hepes buffer only (20 mM Hepes, 149 mM NaCl, pH 7·4). Liposome dispersions were used within 1 week after preparation; they were stored in the refrigerator (4–6 °C).

Liposome characterization

Lipid phosphate was determined by the method of Rouser, Flusher & Yamamoto (1970). Approximately 2% bioactive rhTNF-α appeared to be encapsulated as determined in the WEHI cytotoxicity assay after solubilization of the liposomes (see below). The liposomes used in the experimental studies were non-sized.

Determination of bioactive TNF-α

The bioactivity of rhTNF-α, free or liposome-associated (expressed as dose of free rhTNF-α in the aqueous phase of the liposomes), was determined using the WEHI cytotoxicity assay essentially according to the method described previously (Espevik & Nissen-Meyer, 1986). WEHI 164 clone 13 mouse fibrosarcoma cells were cultured in Iscove's Modified Dulbecco's Medium with glutamine (IMDM; Gibco Life Technologies, Breda, The Netherlands) supplemented with 10% foetal bovine serum (FBS; Integro, Zaandam, The Netherlands), penicillin (100 I.U./ml), streptomycin (100 μg/ml) and amphotericin B (0·25 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. One day before testing, WEHI cells were transferred to new flasks and grown overnight. The next day cells were collected to a final concentration of $0{\cdot}5 \times 10^6$ cells/ml medium of which 50 μl was pipetted into 96-well microtitre plates (Falcon, Micronic, Lelystad, The Netherlands). Following incubation for 3–5 h at 37 °C and 5% CO_2, 50 μl of standard or test sample were added. A standard curve (up to 20000 pg/ml) was made from rhTNF-α (Boehringer Ingelheim, Germany) with an originally defined biological activity of 6×10^7 U/mg in a murine LM bioassay according to WHO standards. Serial dilutions of free TNF-α in medium were added directly to the cells. In case of liposome-associated rhTNF-α, liposomes were disrupted with 10% Triton X–100 in Hepes buffer (10 mM Hepes, 149 mM NaCl, 0·5% bovine serum albumin, pH 7·4) in a volume ratio of 1:1. No effect of Triton X–100 was observed in the bioactivity assay of rhTNF-α with a sample dilution of 30000 times. After incubation for 18 h at 37 °C and 5% CO_2 the viable cells were quantitated using the XTT assay (Scudiero *et al*. 1988). Briefly, 100 μl of *N*-methyldibenzopyrazine methyl sulfate (0·4 mg/ml in phosphate buffered saline) from Sigma (Bornem, Belgium) were added to 5 ml of sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT, Sigma, Bornem, Belgium) and 1 mg/ml RPMI 1640 (Gibco, Life Technologies, Breda, The Netherlands). Then 50 μl of the XTT labelling mixture was added to the wells and incubated for 90 min at 37 °C and 5% CO_2. The absorbance was measured using a Bio-Rad Novapath™ microplate reader (Bio-Rad Laboratories, Veenendaal, The Netherlands) at a wavelength of 495 nm (reference wavelength: 655 nm). The WEHI cytotoxicity assay lower detection limit was approximately 80 pg rhTNF-α/ml.

In the above WEHI cytotoxicity assay, the specific biological activity (U/mg) of rhTNF-α used for the experiments was about 8–10 times higher than for the standard rhTNF-α (Boehringer Ingelheim, Germany).

Mice

Female C57Bl/6J mice, 6–10 weeks old, were obtained from a specific pathogen-free colony main-

https://doi.org/10.1017/S0031182098003552 Published online by Cambridge University Press

tained at the Central Animal Facility of the University of Nijmegen. All mice were housed in plastic cages and received water and standard RMH food (Hope Farms, Woerden, The Netherlands) *ad libitum*.

Parasite and development of experimental cerebral malaria

P. berghei K173 (originally obtained from W. Kretschmar, Tübingen, Germany) has been maintained by weekly transfer of parasitized erythrocyte (PE) from infected into naive mice for more than 30 years. Experimental C57Bl/6J mice were injected i.p. with 10^3 PE from blood of infected donors. About 95 % of C57Bl/6J mice injected i.p. with 10^3 PE die early in the second week after infection (day 9–12) due to the development of ECM as described by Curfs *et al*. (1992). One day before death a progressive hypothermia develops which is strongly correlated with development of haemorrhages in the brain as observed by histology (Polder *et al*. 1992). Mice that survive this critical period show only a limited, transient hypothermia and the majority die in the third week or later after infection with severe anaemia and a parasitaemia of 20–40 %, but without any noticeable cerebral pathology. Day of death after infection was used as the parameter for ECM or protection against ECM in the experiments described in this paper.

Experimental design

For each experiment mice received 10^3 PE i.p. on day 0. At day 5, 6, 7 or 8 after infection, mice were treated with a single s.c. injection of either free rhTNF-α or liposome-associated rhTNF-α. Sustained delivery of rhTNF-α through i.p. implanted mini-osmotic pumps (Alzet model 2001; release rate 1 μl/h for 7 days, Alza, Palo Alto, CA) was started at day 5 or day 7 after infection. Prior to injection, appropriate dilutions were prepared using Hepes buffer (20 mM Hepes, 149 mM NaCl, pH 7·4) containing 1 % mouse plasma.

The effect of treatment on parasitaemia (% infected erythrocytes) at day 8 after infection was studied from thin blood films made from tail-blood and stained with May-Grünwald and Giemsa's solutions. A considerable variation is observed in parasitaemia at day 8 after infection among independent infected control groups (average parasitaemia ± S.D.: 20 ± 15 %; 21 experiments and 3–5 mice/experimental group). Therefore, within each experiment the average parasitaemia in non-treated mice was determined and the parasitaemia of each individual mouse from either a control or a treatment group was divided by this average. These ratios were used to compare and summarize the results of independent repeated experiments.

The effect of treatment on the development of ECM was studied by monitoring survival of mice. Death of mice within 2 weeks after infection was used to identify ECM-death (Curfs *et al*. 1992). Persistent, recurrent parasitaemia and survival for more than 2 weeks after infection indicated protection against ECM.

TNF receptor R55 and R75 (TNFR55 and TNFR75) ELISAs

Mice treated with rhTNF-α and non-treated mice were sacrificed at day 9 after infection to study the effect of treatment on the plasma concentrations of soluble mouse TNF receptors. Blood was collected in endotoxin-free tubes (4 °C) which contained endotoxin-free (LAL assay) heparin (NPBl, Emmer Compascuum, The Netherlands). Plasma was collected by centrifugation within 2 h after bleeding and stored at −20 °C.

The ELISAs recognizing both free and complexed TNF receptors, were carried out with the materials and according to the methods described by Bemelmans *et al*. (1994). Briefly, microtitre plates were coated with anti-TNFR55 or anti-TNFR75 monoclonal antibody. Non-specific binding was blocked with 1 % BSA. After washing, mouse plasma samples were added. A standard curve was made using recombinant murine TNFR55 and TNFR75 protein. Subsequently, plates were washed and incubated with biotinylated anti-TNF receptor antibodies. After washing, plates were incubated with streptavidin–peroxidase followed by an enzyme reaction providing a coloured reaction product. The lower detection limit for murine soluble TNFR55 was 20 pg/ml and for soluble TNFR75 was 500 pg/ml.

Statistical analysis

The effect of treatment on parasitaemia was analysed by one-way ANOVA. The effect of treatment on survival was analysed by the χ^2 test. Differences were considered significant at a level $\alpha = 0{\cdot}05$.

RESULTS

Subcutaneous injection of free rhTNF-α in P. berghei*-infected mice*

Mice received a single s.c. injection of free rhTNF-α on day 5, 6, 7 or 8 after infection and protection against ECM was monitored (Fig. 1). Approximately 50–90 % of the mice were protected against CM when treatment was started at day 5 after infection (0·5–5 μg rhTNF-α). The protective efficacy against ECM depended on the day of treatment (days 5–8, $P < 0{\cdot}0001$; summarized data of all doses) and decreased when treatment was started later in infection. Protection against ECM was independent of the

https://doi.org/10.1017/S0031182098003552 Published online by Cambridge University Press

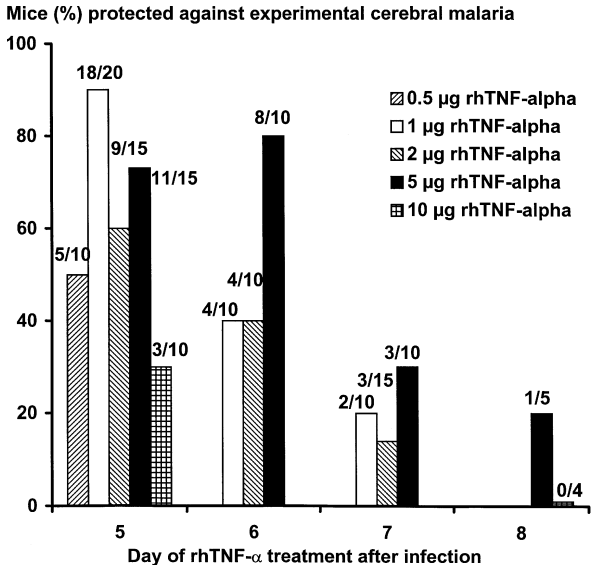

Fig. 1. The effect of treatment with free rhTNF-α during infection on protection against ECM. Mice received a single subcutaneous injection of rhTNF-α (0·5–10 μg rhTNF-α) at day 5, 6, 7 or 8 after infection with 10^3 *Plasmodium berghei* parasites and survival for more than 2 weeks after infection was used as a marker for protection against ECM. The percentage of mice protected against ECM is plotted against the day of treatment and the dose of rhTNF-α. The number above each bar indicates mice protected/mice treated. Non-treated or placebo-treated mice died within 2 weeks after infection.

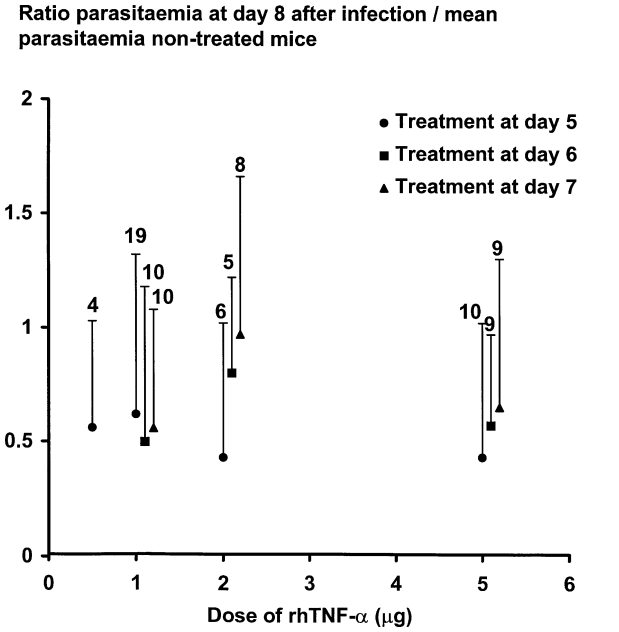

Fig. 2. The effect of treatment with rhTNF-α during infection on parasitaemia. Mice received a single subcutaneous injection of rhTNF-α (0·5–5 μg rhTNF-α) at day 5, 6 or 7 after infection with 10^3 *Plasmodium berghei* parasites. The ratio of parasitaemia at day 8 after infection/mean parasitaemia non-treated mice was calculated for each mouse as described in the Materials and Methods section. The average ratio + S.D. is plotted in the figure for each dose of rhTNF-α and for each day of treatment and the total number of mice in each group is indicated. (The average ratio of parasitaemia $\pm$S.D. at day 8 for non-treated mice: 1·0 $\pm$ 0·6.)

dose of rhTNF-α in the studied dose range 0·5–5 μg rhTNF-α (summarized data of treatment of days 5–7). Administration of 10 μg rhTNF-α was toxic (death within 24 h after injection) at day 5 for 7 out of 10 mice, while all mice died within 24 h when injected at day 8. No toxic effects were observed after injection of 5 μg rhTNF-α at day 8 after infection. Non-treated mice and mice treated with buffer all died within 2 weeks after infection because of ECM.

The parasitaemia data are expressed as the ratio of the parasitaemia of a certain mouse divided by the mean parasitaemia of the infected but otherwise non-treated mice in the same experiment. Calculation of this ratio permitted the results of independent repeat experiments to be summarized. The effect of rhTNF-α treatment on parasitaemia was similar when treatment was started at day 5, 6 or 7 and in the dose-range 0·5–5 μg rhTNF-α (Fig. 2). Therefore, statistical analysis was performed on the summarized data. Treatment with rhTNF-α significantly ($P <$ 0·0001 compared to non-treated mice) suppressed parasitaemia independent of the day of treatment (day 5–7; summarized data of doses ranging from 0·5–5 μg rhTNF-α) and independent of dose of rhTNF-α (0·5–5 μg rhTNF-α; summarized data of treatment on days 5–7) (Table 1). The effect of rhTNF-α treatment on parasitaemia was also studied in relation to protection against ECM; parasitaemia was significantly suppressed in ECM-protected mice but not in mice developing ECM ($P <$ 0·0001, Table 1).

Sustained release of rhTNF-α in P. berghei*-infected mice*

Sustained release of low doses of 0·05–0·15 μg rhTNF-α/day, from i.p. implanted osmotic pumps (release rate 1 μl/h during 7 days) starting at day 5 after infection protected 65–100% of the mice against the development of ECM (Table 2). The protective efficacy against ECM was independent of dose in the dose range studied.

The summarized data of mice receiving saline-containing pumps exhibited protection against ECM in 5 out of 16 mice. This was an unexpectedly high protection rate when considering the outcome of earlier work (unpublished data). For unknown reasons in 1 of the 4 independent experiments 3 out of 3 mice treated with the saline containing pumps were protected against ECM. Despite this, protection against ECM was significantly ($P <$ 0·01) higher by sustained release of rhTNF-α (0·05–0·15 μg rhTNF-α/day) as compared to saline-treated controls.

https://doi.org/10.1017/S0031182098003552 Published online by Cambridge University Press

Table 1. Effect of treatment with a subcutaneous bolus injection of rhTNF-α on parasitaemia in relation to development of experimental cerebral malaria in *Plasmodium berghei*-infected mice

(Mice received a subcutaneous injection rhTNF-α (0·5–5 μg) on day 5, 6 or 7 after infection with 10^3 parasites and the effect on parasitaemia at day 8 after infection was studied in relation to protection against cerebral malaria. The average parasitaemia at day 8 after infection was determined in each control group and the parasitaemia of each individual mouse was divided by the average of the corresponding control group. This permitted the results of independent repeat experiments to be summarized. Values represent averages ± S.D. of the combined data of different doses (0·5–5 μg rhTNF-α) and days of treatment (day 5, 6 or 7).)

	Ratio parasitaemia at day 8/mean parasitaemia non-treated mice		
Treatment	All mice	Mice with ECM	Mice without ECM
No treatment	1 ± 0·6 ($n = 87$)	1 ± 0·6 ($n = 87$)	($n = 0$)
rhTNF-α	0·6** ± 0·6 ($n = 101$)	0·9 ± 0·6 ($n = 48$)	0·4**† ± 0·4 ($n = 53$)

**$P < 0·0001$ as compared to non-treated mice; †$P < 0·0001$ as compared to mice developing ECM in the same treatment group.

Table 2. Effect of sustained release of rhTNF-α from intraperitoneally implanted osmotic pumps on protection against experimental cerebral malaria

(The pumps were implanted at day 5 after infection with 10^3 *Plasmodium berghei* parasites and released 1 μl/h during 7 days. Controls received either pumps containing saline or no treatment at all.)

Treatment	Number of ECM protected mice/number of treated mice
No treatment	2/86 (2%)
Pump	
Saline	5/16 (31%)
0·05 μg rhTNF-α/day	13/20 (65%)
0·10 μg rhTNF-α/day	4/4 (100%)
0·15 μg rhTNF-α/day	8/9 (89%)

Treatment with rhTNF-α via osmotic pumps (0·05–0·15 μg rhTNF-α/day) starting at day 5 after infection significantly ($P < 0·001$) suppressed parasitaemia (average ± S.D.: 0·4 ± 0·3; $n = 14$) (data not shown).

When sustained release of rhTNF-α started at day 7 after infection, no effect was observed on parasitaemia and mice were not protected against ECM (data not shown).

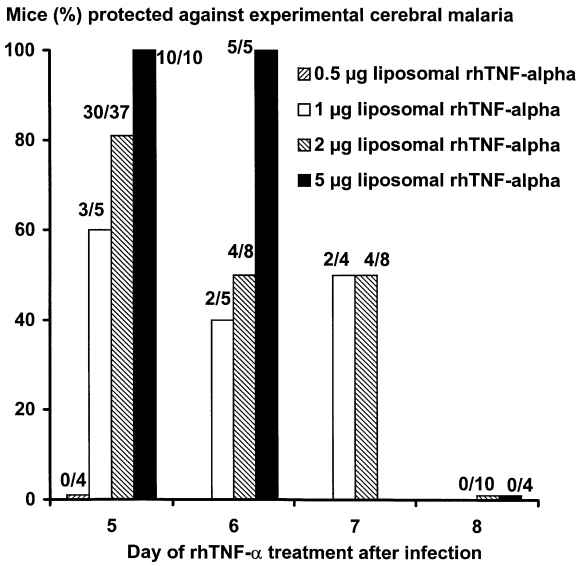

Fig. 3. The effect of treatment with liposomal rhTNF-α during infection on protection against ECM. Mice received a single subcutaneous injection of liposomal rhTNF-α (0·5–5 μg bioactive rhTNF-α) at day 5, 6, 7 or 8 after infection with 10^3 *Plasmodium berghei* parasites and survival for more than 2 weeks after infection was used as a parameter for protection against ECM. The percentage of mice protected against ECM is plotted against the day of treatment and the dose of rhTNF-α administered. The number above each bar indicates mice protected/mice treated. Non-treated or placebo-treated mice died within 2 weeks after infection.

Subcutaneous injection of liposomal rhTNF-α in P. berghei*-infected mice*

Subcutaneous injection of rhTNF-α encapsulated into fluid type (EPC:EPG) liposomes was used as another approach for sustained release of rhTNF-α. The dose range of 0·5–5 μg of bioactive rhTNF-α as used for free rhTNF-α was also used for the liposomal rhTNF-α preparation. Protection against ECM was dose dependent (0·5–5 μg liposomal rhTNF-α, $P < 0·001$; summarized data of treatment on days 5–7) and increased to 100% protection when 5 μg liposomal rhTNF-α was injected at day 5 or 6 after infection (Fig. 3). The protective efficacy of liposomal rhTNF-α against ECM also depended on the day of treatment (day 5–8, $P < 0·0001$; summarized data of all doses) and decreased when treatment was started later in infection, similar to the results obtained with free rhTNF-α (*cf.* Fig. 1 and Fig. 3). Injection of liposomal rhTNF-α at day 8 was completely ineffective, but also non-toxic.

https://doi.org/10.1017/S0031182098003552 Published online by Cambridge University Press

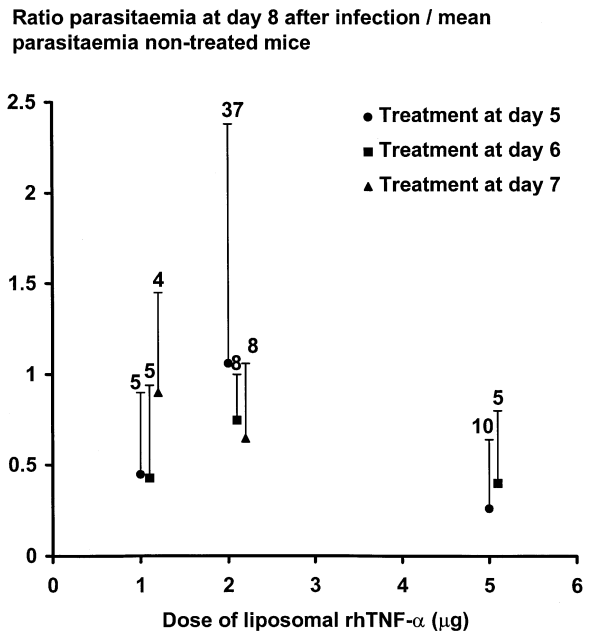

Fig. 4. The effect of treatment with liposomal rhTNF-α during infection on parasitaemia. Mice received a single subcutaneous injection of liposomal rhTNF-α (1–5 μg rhTNF-α) at day 5, 6 or 7 after infection with 10^3 *Plasmodium berghei* parasites. The ratio of parasitaemia at day 8 after infection/mean parasitaemia non-treated mice was calculated for each mouse as described in the Materials and Methods section. The average ratio $\pm$S.D. is plotted in the figure for each dose of liposomal rhTNF-α and for each day of treatment and the total number of mice in each group is indicated. (The average ratio of parasitaemia $\pm$S.D. at day 8 for non-treated mice: $1{\cdot}0 \pm 0{\cdot}6$).

The effect of treatment with liposomal rhTNF-α on parasitaemia as compared with non-treated mice did not reach significance ($P < 0{\cdot}06$), which is probably linked to the remarkably high parasitaemia of a few mice treated with 2 μg liposomal rhTNF-α (Fig. 4 and Table 3). On the other hand, parasitaemia was significantly ($P < 0{\cdot}001$) suppressed in ECM-protected mice as compared to non-treated mice or mice developing ECM (Table 3).

Subcutaneous injection of empty, buffer-loaded liposomes at day 5, 6 or 7 after infection did not protect against ECM (data not shown) and did not affect parasitaemia (Table 3).

Effect of sustained treatment with rhTNF-α on sTNFRs in plasma

The effect of treatment by sustained release of rhTNF-α on the concentrations of sTNFRs in plasma was determined at day 9 after infection when non-treated mice normally die of ECM.

Groups of mice received protective doses of 0·5 μg rhTNF-α/day (mini-osmotic pump) or 2 μg liposomal rhTNF-α s.c. at day 5 after infection. The concentrations of soluble mouse TNFR55 and TNFR75 in rhTNF-α treated and non-treated mice are depicted in Table 4. No differences were observed in the concentration of soluble mouse TNFR55 at day 9 after infection in plasma samples of non-treated and rhTNF-α treated mice. In contrast, soluble mouse TNFR75 concentrations were increased significantly ($P < 0{\cdot}05$) at day 9 after infection when rhTNF-α was released from osmotic pumps, but not when rhTNF-α containing liposomes were administered s.c. at day 5.

DISCUSSION

The most important observation of this study is that low-dose treatment with rhTNF-α protected mice against *P. berghei* K173 experimental cerebral malaria and suppressed parasitaemia when treatment was started at day 5 or 6 after infection. The rhTNF-α mediated protection against ECM gradually disappeared when treatment was postponed to day 7 or 8 after infection. The same protective efficacy was obtained by either s.c. injection of free or liposome-encapsulated rhTNF-α or i.p. infusion, but a 10 times lower daily dose of rhTNF-α was sufficient when continuously released from an i.p. implanted osmotic pump.

Sustained release of rhTNF-α probably results in prolonged therapeutic concentrations of rhTNF-α in the circulation. Recombinant human TNF-α is rapidly cleared from the circulation after i.v. injection into mice with an initial half-life less than 20 min and a subsequent slower β-phase (Ferraiolo *et al*. 1988). The sustained effect of rhTNF-α released from osmotic pumps is also shown by the higher plasma concentrations of sTNFR75 as compared with non-treated or liposomal rhTNF-α treated mice.

The observation that doses needed for protection against ECM by treatment with liposomal rhTNF-α were as high as for free rhTNF-α and the observation that sTNFR75 was not increased by this treatment, suggest that the s.c. liposomal depot released protective amounts of rhTNF-α only for a limited period of time.

The mortality observed after s.c. injection of 10 μg rhTNF-α at day 5 or 8 probably reflects the role of TNF-α in malaria pathology as has been described before (Curfs *et al*. 1993) and limits the dose that can be administered by s.c. bolus injection.

A significant suppression of parasitaemia by rhTNF-α treatment was observed in ECM-protected mice. Under the experimental conditions used, the reduction of parasitaemia was independent of day of treatment or treatment regimen. Suppression of *P. chabaudi* AS parasitaemia by administration of rhTNF-α to mice was observed by Stevenson & Ghadirian (1989), while TNF-α alone did not inhibit growth of *P. yoelii in vitro* (Taverne *et al*. 1987). Studies with *P. falciparum in vitro*

https://doi.org/10.1017/S0031182098003552 Published online by Cambridge University Press

Table 3. Effect of treatment with a subcutaneous injection of liposomal rhTNF-α on parasitaemia in relation to development of experimental cerebral malaria in *Plasmodium berghei*-infected mice

(Mice received a subcutaneous injection of empty liposomes or liposome-encapsulated rhTNF-α (1–5 μg rhTNF-α) at day 5, 6 or 7 after infection with 10^3 parasites and the effect on parasitaemia at day 8 after infection was studied in relation to protection against cerebral malaria. The average parasitaemia at day 8 after infection was determined in each control group and the parasitaemia of each individual mouse was divided by the average of the corresponding control group. The ratios were used for further analysis. Values represent averages ± S.D. of the combined data of different doses (1–5 μg rhTNF-α) and days of treatment (day 5, 6 or 7).)

	Ratio parasitaemia at day 8/mean parasitaemia non-treated mice		
Treatment	All mice	Mice with ECM	Mice without ECM
No treatment	1·0 ± 0·6 ($n = 86$)	1·0 ± 0·6 ($n = 86$)	($n = 0$)
Empty liposomes	0·8 ± 0·3 ($n = 15$)	0·8 ± 0·3 ($n = 15$)	($n = 0$)
Liposomal rhTNF-α	0·8 ± 1·0 ($n = 82$)	1·5* ± 1·3 ($n = 23$)	0·5*† ± 0·6 ($n = 59$)

*$P < 0·01$ as compared to non-treated mice; †$P < 0·0001$ as compared to mice developing ECM in the same treatment group.

Table 4. Effect of sustained rhTNF-α treatment starting at day 5 after infection on the concentrations of soluble mouse TNF receptor in plasma at day 9 after infection in *Plasmodium berghei*-infected mice

(Mice received rhTNF-α either from i.p. implanted mini-osmotic pumps (release rate 1 μl/h, 0·5 μg rhTNF-α/day for 7 days) or from a single s.c. injection of 2 μg liposomal rhTNF-α at day 5 after infection with 10^3 parasites. A third group of mice did not receive rhTNF-α treatment. Groups of mice were sacrificed at day 9 after injection and plasma was collected for analysis by specific ELISAs. Values represent averages ± S.D.)

	Soluble mouse TNF receptor in plasma (ng/ml) at day 9 after infection	
Treatment	sTNFR55	sTNFR75
No treatment	0·3 ± 0·2 ($n = 5$)	50 ± 9 ($n = 5$)
rhTNF-α infusion (0·5 μg/day)	< 0·2 ($n = 3$)	84* ± 13 ($n = 3$)
Liposome-encapsulated rhTNF-α (2 μg)	0·3 ± 0·1 ($n = 5$)	54 ± 13 ($n = 5$)

*$P < 0·05$ as compared to non-treated mice.

(Kumaratilake, Ferrante & Rzepczyk, 1990) and *P. yoelii* and *P. chabaudi* AS in mice (Taverne *et al.* 1994) indicate that activated macrophages and neutrophils are probably involved in TNF-α mediated parasite killing. In addition, Taverne *et al.* (1994) suggested that the effects of TNF-α on erythropoiesis (Johnson *et al.* 1989; Moldawer *et al.* 1989) may play a role in the suppression of parasitaemia.

The observation that parasitaemia was suppressed in ECM-protected mice, but not in mice developing ECM, suggests that the effect of rhTNF-α on parasitaemia plays a role in rhTNF-α mediated protection against ECM. It should be noted that almost all mice infected with parasites in a range of 10 to 10000, develop ECM despite a widely varying parasitaemia after infection (Curfs *et al.* 1993). Moreover, parasitaemia in non-treated mice varies extensively before the mice die of ECM. Therefore, the course of an untreated infection is probably not a critical factor in development of ECM in *P. berghei*-infected mice. Thus, the change in parasitaemia by treatment with rhTNF-α may interfere with the development of ECM. On the other hand, the effect of rhTNF-α on parasitaemia may be an associated but not causally related factor in protection against ECM.

Possible other mechanisms involved in the rhTNF-α mediated protection are the stimulation of the production of sTNFRs and the induction of tolerance or refractoriness of TNF-α sensitive cells. Elevated concentrations of sTNFRs have been observed during malaria infection in humans (Deloron *et al.* 1994) and in the *P. berghei* K173 murine model used in the present study (Hermsen *et al.* 1997). Soluble TNFRs are probably increased as a response to endogenous TNF-α production. They can inhibit TNF-α interaction with membrane-

https://doi.org/10.1017/S0031182098003552 Published online by Cambridge University Press

bound receptors (Lesslauer *et al*. 1991) and high levels of sTNFR55 were shown to protect against CM (Garcia *et al*. 1995). Our study did not reveal an increase in the plasma concentration of sTNFR55 in relation to sustained treatment with rhTNF-α. The increase in sTNFR75 at day 9 after infection is only observed in mice receiving rhTNF-α from osmotic pumps, not in mice receiving rhTNF-α from a s.c. liposomal depot, while both treatment regimens protected against ECM. This suggests that the increase in sTNFR75 is not linked to protection against ECM. However, only one time-point was measured and that may not be the critical point to observe functional changes in sTNFR75 involved in the protection against ECM.

Another mechanism involved in rhTNF-α mediated protection against ECM may be the induction of refractoriness or tolerance of TNF-α sensitive cells. Several studies demonstrated that pre-treatment with TNF-α induces refractoriness and therefore tolerance to its pathological effects (Fraker *et al*. 1988; Takahashi *et al*. 1995). Tolerance persists for several days and might be a 'natural' regulatory mechanism to control toxicity of this cytokine (Takahashi, Brouckaert & Fiers, 1993). Our data do not permit a decision as to whether treatment with rhTNF-α induces refractoriness and therefore prevents development of ECM.

In summary, low-dose treatment with rhTNF-α at day 5 or 6 after infection protects against ECM in *P. berghei* K173-infected mice. The effect of rhTNF-α treatment on suppression of parasitaemia may play a role. No indications were found that treatment with rhTNF-α protects through triggering of production of sTNFRs. The mechanism by which rhTNF-α prevents the development of ECM remains to be elucidated.

We thank Dr M. Hora (Chiron Corporation, Emeryville, CA) for the generous gift of rhTNF-α. We also gratefully thank Dr W. Buurman (Department of General Surgery, University of Limburg, The Netherlands) for supplying us with the materials used for the sTNF receptor ELISAs and Prof. Dr. J. van de Meer (University Hospital Nijmegen, Nijmegen, The Netherlands) for the gift of rhTNF-α used as a standard in the WEHI cytotoxicity assay. We thank G. Poelen, T. van de Ing and Y. Brom for their skilled biotechnical assistance.

REFERENCES

ALEXANDER, H. R., SHEPPARD, B. C., JENSEN, J. C., LANGSTEIN, H. N., BURESH, C. M., VENZON, D., WALKER, E. C., FRAKER, D. L., STOVROFF, M. C. & NORTON, J. A. (1991). Treatment with recombinant human tumour necrosis factor-alpha protects rats against the lethality, hypotension, and hypothermia of gram-negative sepsis. *Journal of Clinical Investigation* **88**, 34–39.

BEMELMANS, M. H., ABRAMOWICZ, D., GOUMA, D. J., GOLDMAN, M. & BUURMAN, W. A. (1994). *In vivo* T cell activation by anti-CD3 monoclonal antibody induces soluble TNF receptor release in mice. Effects of pentoxifylline, methylprednisolone, anti-TNF and anti-IFN-gamma antibodies. *Journal of Immunology* **153**, 499–506.

CLARK, I. A. (1978). Does endotoxin cause both the disease and parasite death in acute malaria and babesiosis? *Lancet* **1**, 75–77.

CREASEY, A. A., DOYLE, L. V., REYNOLDS, M. T., JUNG, T., LIN, L. S. & VITT, C. R. (1987). Biological effects of recombinant human tumour necrosis factor and its novel muteins on tumour and normal cell lines. *Cancer Research* **47**, 145–149.

CURFS, J. H. A. J., HERMSEN, C. C., MEUWISSEN, J. H. E. TH. & ELING, W. M. C. (1992). Immunization against cerebral pathology in *Plasmodium berghei*-infected mice. *Parasitology* **105**, 7–14.

CURFS, J. H. A. J., HERMSEN, C. C., KREMSNER, P., NEIFER, S., MEUWISSEN, J. H. E. TH., VAN ROOYEN, N. & ELING, W. M. C. (1993). Tumour necrosis factor-α and macrophages in *Plasmodium berghei*-induced cerebral malaria. *Parasitology* **107**, 125–134.

DELORON, P., ROUX-LOMBARD, P., RINGWALD, P., WALLON, M., NIYONGABO, T., AUBRY, P., DAYER, J. M. & PEYRON, F. (1994). Plasma levels of TNF-alpha soluble receptors correlate with outcome in human falciparum malaria. *European Cytokine Network* **5**, 331–336.

ENGELMANN, H., NOVICK, D. & WALLACH, D. (1990). Two tumour necrosis factor-binding proteins purified from human urine. Evidence for immunological cross-reactivity with cell surface tumour necrosis factor receptors. *Journal of Biological Chemistry* **265**, 1531–1536.

ESPEVIK, T. & NISSEN-MEYER, J. (1986). A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumour necrosis factor from human monocytes. *Journal of Immunological Methods* **95**, 99–105.

FERRAIOLO, B. L., MOORE, J. A., CRASE, D., GRIBLING, P., WILKING, H. & BAUGHMAN, R. A. (1988). Pharmacokinetics and tissue distribution of recombinant tumour necrosis factor-α in mice. *Drug Metabolism and Disposition* **16**, 270–275.

FRAKER, D. L., STOVROFF, M. C., MERINO, M. J. & NORTON, J. A. (1988). Tolerance to tumour necrosis factor in rats and the relationship to endotoxin tolerance and toxicity. *Journal of Experimental Medicine* **168**, 95–105.

GARCIA, I., MIYAZAKI, Y., ARAKI, K., ARAKI, M., LUCAS, R., GRAU, G. E., MILON, G., BELKAID, Y., MONTIXI, C., LESSLAUER, W. & VASSALLI, P. (1995). Transgenic mice expressing high levels of soluble TNF-R1 fusion protein are protected from lethal septic shock and cerebral malaria, and are highly sensitive to *Listeria monocytogenes* and *Leishmania major* infections. *European Journal of Immunology* **25**, 2401–2407.

GRAU, G. E., PIGUET, P.-F., VASSALLI, P. & LAMBERT, P.-H. (1989). Tumour-necrosis factor and other cytokines in cerebral malaria: Experimental and clinical data. *Immunological Reviews* **112**, 49–70.

HERMSEN, C. C., CROMMERT VAN DE, J., FREDERIX, H., SAUERWEIN, R. W. & ELING, W. M. C. (1997). Circulating tumour necrosis factor α is not involved in the development of cerebral malaria in *Plasmodium berghei*-infected C57Bl mice. *Parasite Immunology* **19**, 571–577.

JACOBS, P., RADZICH, D. & STEVENSON, M. (1996). A Th1-associated increase in tumour necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice. *Infection and Immunity* **64**, 535–541.

JOHNSON, R. A., WADDELOW, T. A., CARO, J., OLIFF, A. & ROODMAN, G. D. (1989). Chronic exposure to tumour necrosis factor *in vivo* preferentially inhibits erythropoiesis in nude mice. *Blood* **74**, 130–138.

KADIR, F., ZUIDEMA, J. & CROMMELIN, D. J. A. (1993). Liposomes as drug delivery systems for intramuscular and subcutaneous injections. In *Particulate Drug Carriers in Medical Applications* (ed. Rolland, A.), pp. 165–198. Marcel Dekker Inc., New York.

KUMARATILAKE, L. M., FERRANTE, A. & RZEPCZYK, C. M. (1990). Tumour necrosis factor enhances neutrophil-mediated killing of *Plasmodium falciparum*. *Infection and Immunity* **58**, 788–793.

KWIATKOWSKI, D., HILL, A. S. V., SAMBOU, I., TWUSAMI, P., CASTRANCE, J., MANOGUE, K. R., CERAMI, A., BREWSTER, D. R. & GREENWOOD, B. M. (1990). TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet* **336**, 1201–1204.

LESSLAUER, W., TABUCHI, H., GENTZ, R., BROCKHAUS, M., SCHLAEGER, E. J., GRAU, G., PIGUET, P.-F., POINTAIRE, P., VASSALLI, P. & LOETSCHER, H. (1991). Recombinant soluble tumour necrosis factor receptor proteins protect mice from lipopolysaccharide-induced lethality. *European Journal of Immunology* **21**, 2883–2886.

MOLDAWER, L. L., MARANO, M. A., WEI, H., FONG, Y., SILEN, M. L., KUO, G., MAHOGUE, K. R., VLASSARA, H., COHEN, H. & CERAMI, A. (1989). Cachectin/tumour necrosis factor-α alters red blood cell kinetics and induces anaemia *in vivo*. *FASEB Journal* **3**, 1637–1643.

PEETERS, P. A. M., HUISKAMP, C. W. E. M., ELING, W. M. C. & CROMMELIN, D. J. A. (1989). Chloroquine containing liposomes in the chemotherapy of murine malaria. *Parasitology* **98**, 381–386.

POLDER, T. W., ELING, W. M. C., CURFS, J. H. A. J., JERUSALEM, C. R. & WIJES-ROUW, M. (1992). Ultrastructural changes in the blood-brain barrier of mice infected with *Plasmodium berghei*. *Acta Leidensia* **60**, 31–46.

ROUSER, G., FLUSHER, S. & YAMAMOTO, A. (1970). Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5**, 494–496.

SCUDIERO, D. A., SHOEMAKER, R. H., PAULL, K. D., MONKS, A., TIERNEY, S., NOFZIGER, T. H., CURRENS, M. J., SENIFF, D. & BOYD, M. R. (1988). Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumour cell lines. *Cancer Research* **48**, 4827–4833.

STEVENSON, M. M. & GHADIRIAN, E. (1989). Human recombinant tumour necrosis factor alpha protects susceptible A/J mice against lethal *Plasmodium chabaudi* AS infection. *Infection and Immunity* **57**, 3936–3939.

TAKAHASHI, N., BROUCKAERT, P. & FIERS, W. (1993). Cyclooxygenase inhibitors prevent the induction of tolerance to the toxic effects of tumour necrosis factor. *Journal of Immunotherapy* **14**, 16–21.

TAKAHASHI, N., BROUCKAERT, P. & FIERS, W. (1995). Mechanism of tolerance to tumour necrosis factor: receptor-specific pathway and selectivity. *American Journal of Physiology* **269**, 398–405.

TAVERNE, J., TAVERNIER, J., FIERS, W. & PLAYFAIR, J. H. L. (1987). Recombinant tumour necrosis factor inhibits malaria parasites *in vivo* but not *in vitro*. *Clinical and Experimental Immunology* **67**, 1–4.

TAVERNE, J., SHEIKH, N., DE SOUZA, J. B., PLAYFAIR, J. H. L., PROBERT, L. & KOLLIAS, G. (1994). Anaemia and resistance to malaria in transgenic mice expressing human tumour necrosis factor. *Immunology* **82**, 397–403.

WANG, A. M., CREASEY, A. A., LADNER, M. B., LIN, L. S., STRICKLER, J., VAN ARSDELL, J. N., YAMAMOTO, R. & MARK, D. F. (1985). Molecular cloning of the complementary DNA for human tumour necrosis factor. *Science* **228**, 149–154.