THE ROLE OF NITRIC OXIDE AND ITS UP/DOWNSTREAM MOLECULES IN MALARIA: CYTOTOXIC OR PREVENTIVE?

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Abstract. The current study investigated the involvement of nitric oxide (NO) and related molecules in malaria target organs of outbred MF1 mice during lethal *Plasmodium berghei* and non-lethal *P. c. chabaudi* infections, in order to evaluate whether changes in NO production are beneficial or detrimental to the host. A number of methods have been applied to test this hypothesis, including Griess microassay, electrochemical assay, RT-PCR and Western blot. The results show that reactive nitrogen intermediate (RNI) accumulation, *in vitro* levels of endogenous NO production, inducible nitric oxide synthase (iNOS) mRNA induction and NOS protein expression altered during murine malaria. The changes depended upon the tissue, the day of infection, the degree of parasitemia, the strain of Plasmodia and the method of measuring NO biosynthesis. Differences in the pathology of two strains of Plasmodia appear to depend more on the strain of parasite rather than the strain of host. The involvement of NO and its up/downstream molecules in murine malaria are specified to host/parasite combinations and it is influenced by the method used to assess NO. The anti-parasitic function against Plasmodia did not relate only to NO in this study, but a complex process consisting of NO and other immune factors is required to resolve the parasite. Selective delivery of inhibitors and donors of NO synthesis in the tissues of the malarial host is indicated as a potential novel therapy to inhibit the parasite or prevent its pathological symptoms.

INTRODUCTION

Nitric oxide (NO) is thought to be an important mediator (Bogdan *et al*, 2000), or critical signalling molecule, of malaria immunopathology and a target for novel drug therapy (Taylor *et al*, 1997). NO in the serum of malaria-tolerant people is generated by macrophages and appears to be responsible for malaria tolerance (Clark *et al*, 1996). NO also is reported to inhibit both the liver and blood forms of malaria parasites (Rockett *et al*, 1992). It has been suggested that a cascade of reactions leading to NO production is involved in the killing of infected hepatocytes (Hommel, 1996). *Plasmodium falciparum* malaria has been shown to be susceptible *in vitro* to reactive nitrogen intermediates (RNI), which were toxic in milli-molar (mM) concentrations (Rocket *et al*, 1991). One of the important phenomena related to parasite damage within erythrocytes is the 'crisis form', which is induced by macrophage-derived toxic products (Collier *et al*, 1998). Both reactive oxygen intermediates (ROI) and NO are significantly involved in the formation of the 'crisis form' in parasitized red blood cells (PRBC) (Hommel, 1996). However, there are some contradictory reports about the role of NO

and related molecules in malaria (Chiwakata *et al*, 2000). Data reported by several researchers do not support a potent role for NO during malaria. NO does not play an anti-parasitic role in *P. yoelii* infection (Amante and Good, 1997). Some evidence in iNOS-deficient mice indicated that NO production is not a crucial factor for the development of murine cerebral malaria (Favre *et al*, 1999), but higher parasitemias were found in iNOS-deficient C57BL/6 mice infected with *P. chabaudi* (Balmer *et al*, 2000).

NO production in the resistance to malaria might be strain specific. This might explain some of the contradictory data obtained with different human malaria studies; NO correlated with cerebral malaria in Papua New Guinea (Al Yaman *et al*, 1996), but an inverse correlation was observed in Tanzania (Anstey *et al*, 1996), which is an indication of host specificity and NO.

The aim of this study was to investigate the involvement of NO and related molecules in target organs (blood, brain, liver, spleen) of MF1 mice during lethal *P. berghei* and non-lethal *P. c. chabaudi* malaria.

MATERIALS AND METHODS

Animals

Mice used in this study were male outbred MF1 supplied by the Biological Services Unit, University of Manchester. Animals were housed in plastic cages

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(length 38 cm × width 22 cm × height 11cm) at room temperature 19˚-22˚C, on a 12 hour light (0800-2000) and 12 hour dark (2000-0800) cycle, with unlimited access to food (CRM feeding pellets, SDS) and tap water. Experiments were licensed under the Animals (UK Scientific Procedures) Act 1986. In compliance with the conditions of this license, infected animals were humanely killed at the onset of the terminal phase of malaria.

Malaria parasites

P. berghei N/13/1A/4/203 originally obtained from the School of Tropical Medicine, University of Liverpool, UK and *P. c. chabaudi* AS strain kindly donated by Dr H Helmby, Department of Immunology, The University of Manchester, UK were used in this study. Both parasites were maintained by blood passage in MF1 mice when active parasites were required; otherwise they were stored at -70˚C in Alserver's solution (2.33% glucose, 0.525% NaCl and 1% sodium citrate in deionized water) and glycerol (9:1 parts by volume).

Inoculation of malaria parasites

Mice were inoculated (0.2 ml, i.v.) into a tail vein with blood from a donor mouse (41% parasitemia *P. berghei,* 34% parasitemia *P. c. chabaudi*) diluted with 0.85% saline to contain 2×10^7 PRBC. Control groups received an equivalent volume (0.2 ml/mouse, i.v.) of diluted uninfected red blood cells (URBC).

Parasitemia

In all animals, parasitemia was determined on different days after infection using blood smears stained with Leishman's reagent (Sigma Chemical Co, USA) 2 mg/ml methanol. PRBC were counted in five different fields, each of approximately 200 cells. Results are expressed as the mean percentage (%) of erythrocytes containing Leishman positive bodies.

Griess microassay

Mice were terminally anesthesized by inhalation of diethyl ether (BDH, England) and blood taken by cardiac puncture into a 1 ml syringe containing 50 i.u. heparin (Monoparin, CP Pharmaceuticals Ltd, Wareham, UK). Plasma was prepared by centrifuging blood at 1,500 RCF (relative centrifugal force) (MSE Centaur 2, UK) for 10 minutes and stored at -70˚C until assayed. After blood collection, animals were humanely sacrificed by cervical dislocation and the brain, liver and spleen removed. Tissues were weighed, placed in separate 1.5 ml microfuge tubes and homogenized in ice-cold deionized water (0.1g wet tissue/1ml water) using an electrical homogenizer

(Model RS541-242, RS Components, Corby, UK). Homogenates were centrifuged at 13,400 RCF (Model 1-13 Microcentrifuge, Sigma, Germany) for 15 minutes. RNI were measured in supernatant fluids using the Griess reaction, after first converting nitrates to nitrites with nitrate reductase treatment. The Griess reaction was adapted, with modifications, from the methods of Amante and Good (1997) and Rockett *et al* (1994) to assay nitrite. RNI was determined indirectly by the Griess assay, as the nitrite produced from nitrate when incubated with nitrate reductase. Sixty microliters samples were treated with 10 µl nitrate reductase [NAD(P)H *Aspergillus* species 5U/ml, Sigma Chemical Co, UK] and 30 µl NADPH β-nicotinamide adenine dinucleotide phosphate (1.25 mg/ml, Sigma Diagnostics, St Louis, USA). Two hundred microliters Griess reagent [5% phosphoric acid, 1% sulfanilic acid and 0.1% N (1-naphthyl-1)-ethylenediamine dihydrochloride (NED), all from Sigma Chemical Co, UK, dissolved in 100 ml deionized water] was then added and proteins subsequently precipitated by 200 µl trichloroacetic acid 10%, (BDH, England). Tube contents were vortex mixed, then centrifuged at 13,400 RCF (Model 1-13 Microcentrifuge, Sigma, UK). Duplicate 200 µl samples of supernatants were transferred to a 96-well flat-bottomed microplate (Costar, USA) and absorbances read at 520 nm using a microplate reader (Dynatech, MRX, USA).

Electrochemical assay

Blood was taken as previously described and, following humane killing, tissue samples (brain, liver, spleen) were removed and placed in 10ml Krebs-Henseleit solution (composition in g/l: NaCl 6.92, KCl 0.35, $CaCl_2.6H_2O$ 0.28, $MgSO_4.7H_2O$ 0.29, KH_2PO_4 0.16, $NaHCO_3$ 2.1 and glucose 2.1; all reagents from Sigma Chemical, Co, UK), gassed with 95% O_2 and 5% CO_2, filtered via pore size 22 µm (Millipore Corporation, USA) and stored at 4˚C. Weighed samples of tissues (approximately 0.1g) were placed in 1.5 ml microfuge tubes and homogenized using an electrical homogenizer (Model RS541-242, Refer Scientific, UK) with the gradual addition of 1ml Krebs-Henseleit solution. Homogenates were incubated at room temperature (19˚-22˚C) for 10 minutes and then assayed for NO production using an electrically isolated NO potentiostat. An isolated NO meter with a 200 µm sensor tip (*ISO-NO* Mark II World Precision Instruments, Sarasota, Florida, USA) was calibrated with $NaNO_2$ standard solutions and applied according to the manufacturer's instructions. NO detection *in vitro* was effected according to the method of Rysz *et al* (1997) with the modification of using tissue

homogenates. The NO sensor probe was immersed in suspensions of tissue homogenates incubated at room temperature (19˚-22˚C) and NO production measured continuously until each record returned to baseline values. A PC-based data acquisition system (Duo-18, World Precision Instruments, USA) was used to record and display NO concentrations.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from brain, liver and spleen using total RNA extraction reagent, TRIZOL (GIBCO$_{BRL}$, Life Technologies, UK). Reverse transcription was then applied using Omniscript Reverse Transcriptase (QIAGEN Ltd, UK). Amplification of the cDNA was performed using specific primers for iNOS (inducible NOS) and β-actin (Genosys Biotechnologies Ltd, Cambridge, UK) for 34 cycles (denaturing 95˚C 30 seconds, annealing 69˚C 30 seconds, extension 72˚C 20 seconds) in a Thermal MiniCycler^{TM} (Genetic Research Instrumentation Ltd, Essex, UK). Equivalent aliquots of each amplification reaction and DNA molecular weight marker (Boehringer Mannheim, GmbH, Germany) were separated on a 2% agarose gel which were then electrophoresed for 40 minutes at 80V. Predicted PCR products for iNOS and β-actin were, respectively, 355 and 605 base pairs. Optical densities (OD) of bands were measured using the Scion Image program.

Western blotting (WB)

Tissues (blood, brain, liver, spleen) were removed under terminal anesthesia, homogenized with lysis buffer, and proteins were determined with Bradford assay. Samples were then boiled for 5 minutes at 95˚C in sample buffer, 10 μg of each protein was separated by SDS-PAGE 8%, transferred electrophoretically to a nitrocellulose membrane 0.45 μm (Bio-Lab Laboratories, USA), probed with a rabbit polyclonal anti-iNOS Ab followed by a goat anti-rabbit IgG conjugated with HRP (horseradish peroxidase) (both from Transduction Laboratory, Lexington, USA) directed to LumiGLO® Chemiluminescent Substrate (New England Biolabs Co, UK) and finally detected in a hyper film by film processor (Kodak, X-OMAT M35, Co, UK).

Statistical analysis

Values for NO production, RNI concentration, NOS (nitric oxide synthase) expression and iNOS mRNA induction are presented as the mean ± SEM for groups of n=4-5 mice. The significance of differences was determined by Student's t-test using GraphPad Prism Software (GraphPad, San Diego, California, USA) (*p<0.05, **p<0.01, ***p<0.001).

RESULTS

The results show RNI accumulation (Fig 1), *in vitro* levels of NO production (Fig 2), NOS protein expression (Fig 3) and iNOS mRNA induction (Fig 4), all altered during murine malaria. The changes depended upon the tissue, the day of infection, the degree of parasitemia, the strain of Plasmodia and the method of NO assay.

DISCUSSION

Despite the importance of NO as a biological mediator, few methods have been described for its assay. Electrochemical detection is sensitive (nM concentrations) and measures NO directly, but it is technically more demanding, expensive and, because of the short biological life of NO, has to be applied at the site of NO production. An alternative assay uses the Griess reaction to measure nitrite, which, if applied after catalytic reduction of nitrates in samples, measures both free nitrite and the larger nitrate pool. These values are commonly used as a measure of RNI (White, 1998; Nahrevanian and Dascombe, 2001). The Griess assay is less sensitive than electrochemical detection, but it is cheap and can be applied to the micromolar concentrations of relatively stable NO metabolites that accumulate both locally and away from the site of NO production, the identity of which may be unknown. For such reasons as these, the Griess assay has been central in determining the involvement of NO in malaria (White, 1998).

The involvement of NO and its up/downstream molecules in murine malaria may relate to specific host/parasite combinations (Balmer *et al*, 2000). The evidence is also influenced by the method used to assess NO (Nahrevanian and Dascombe, 2001). The anti-parasitic function against Plasmodia may not relate only to NO, but to a complex process involving other immune factors (White, 1998). Taken together, the results presented here, and the data provided by others, highlight the fact that NO and/or its related molecules are involved in malaria, but the involvement may not be independent of other immune events. It is indicated that NO is an important, but possibly not essential, contributor in the control of acute phase malaria infection. Although, the protective immune responses against the malaria parasite is multifactorial and the final effector molecules that mediate parasite death are not known, NOS, NO and RNI have been significantly implicated (Nahrevanian and Dascombe, 2001).
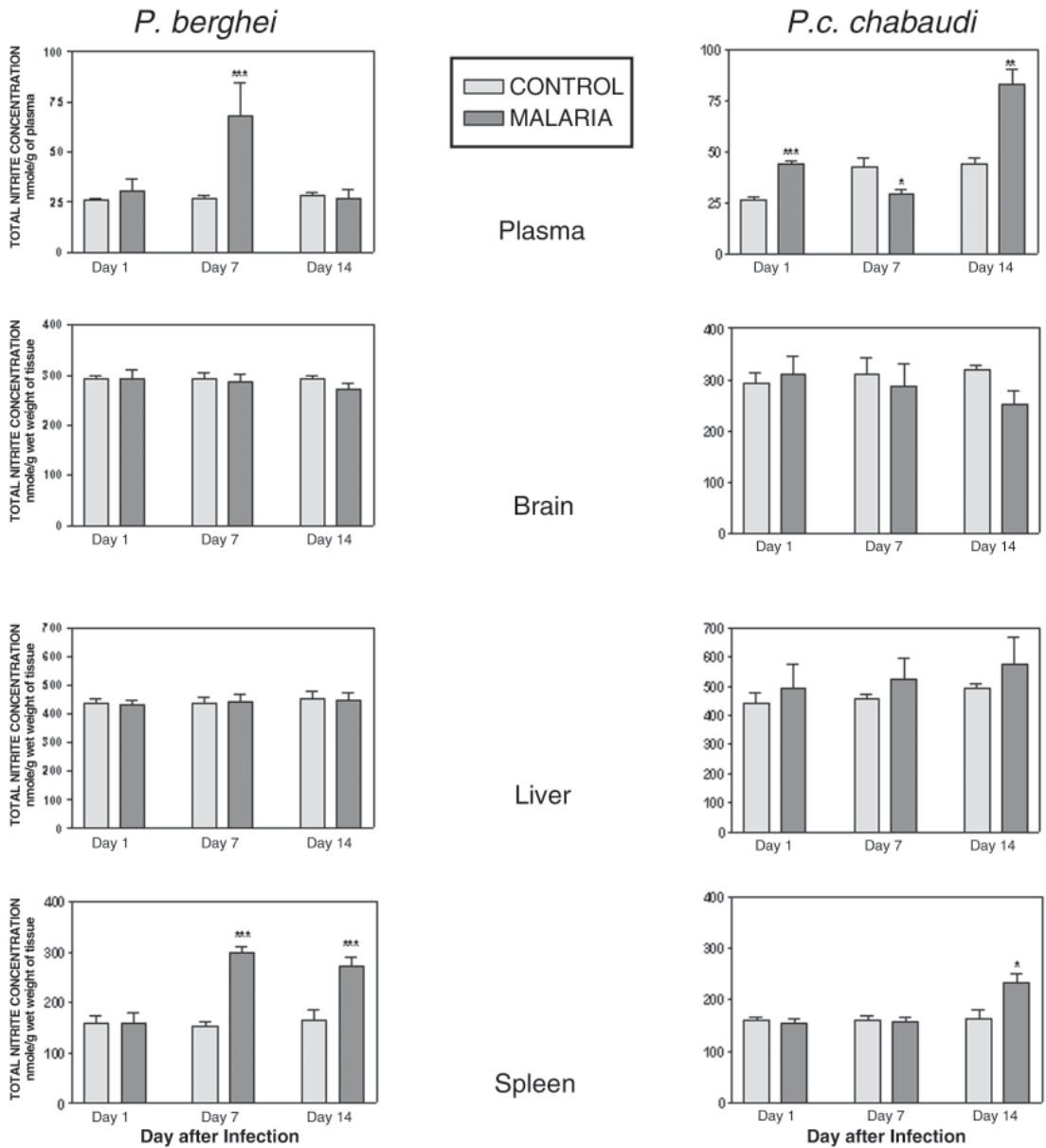
Fig 1- Comparison of RNI concentrations in target organs of *P. berghei* and *P. c. chabaudi* infected mice.
Mean ± SEM, n=5, *p<0.05, **p<0.01, ***p<0.001, Student's *t*-test.

Conclusion

It is concluded that NO is only part of an immuno-pathological chain against malaria infection and the anti-parasitic function against Plasmodia did not relate only to NO action. Therefore, a combination of NO and other immune factors is required to resolve Plasmodia and to eliminate malaria in the host (Good and Doolan, 1999). Perhaps, NO comes from several cellular sources, which can contribute towards the protective immune responses against intracellular Plasmodia. Further investigation in defining these sources will be important for the understanding of cell-mediated defense mechanism(s) in malaria. Selective delivery of inhibitors and donors of NO synthesis in tissues of the malarial host are also indicated as potential novel therapies to inhibit the parasite or prevent its pathological symptoms.
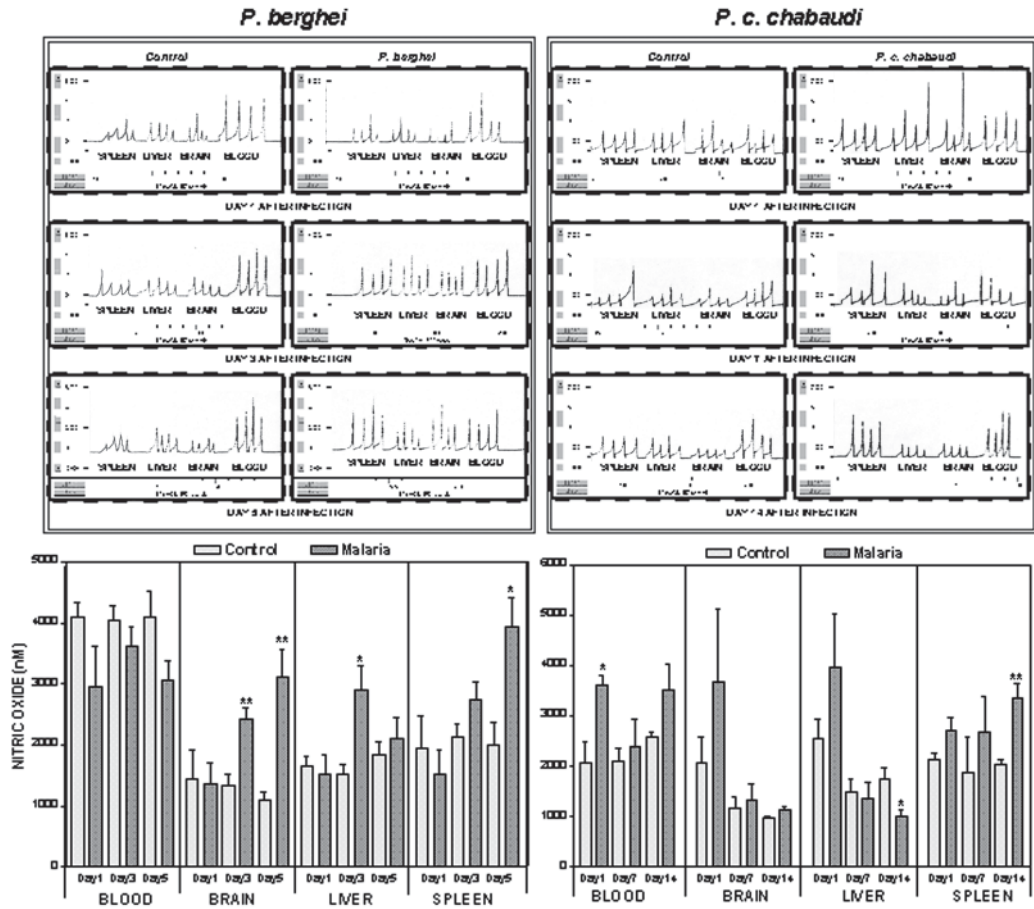
Fig 2- *In vitro* electochemical direct detection of NO in the target organs of *P. berghei* and *P. c. chabaudi* infected mice.
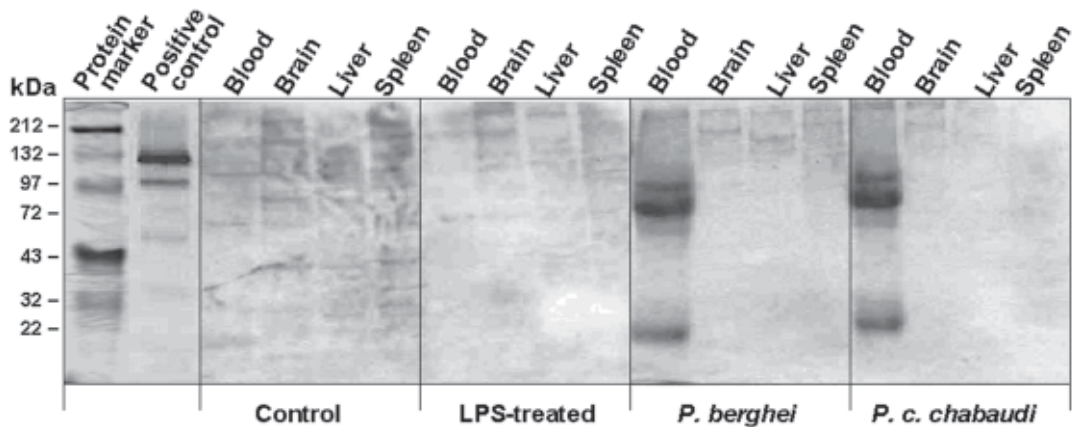Mean ± SEM, n=4, *p<0.05, **p<0.01, Student's *t*-test.

Fig 3- NOS protein expression in target organs of murine malaria.
Control (URBC inoculated), LPS (0.5 mg/kg iv), *P. berghei* (Day 4) and *P. c. chabaudi* (Day 7).

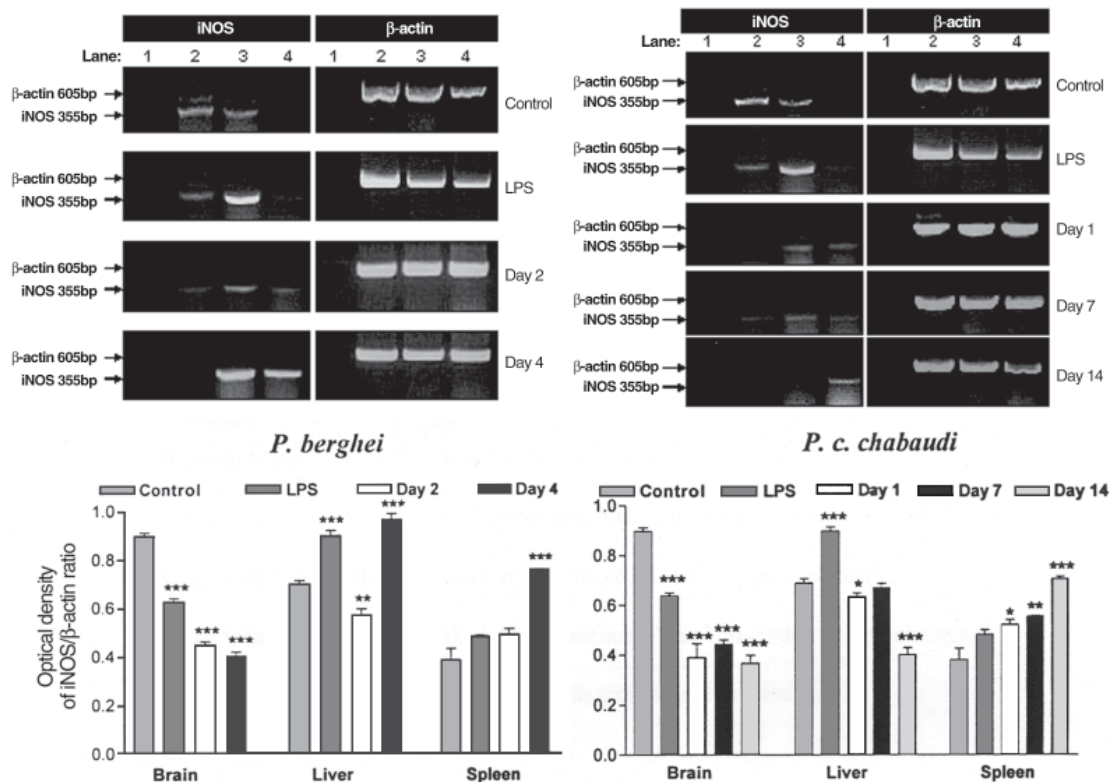## 1) Negative control, 2) Brain, 3) Liver, 4) Spleen

Fig 4- RT-PCR amplification of iNOS mRNA in target organs of *P. berghei* and *P. c. chabaudi* infected mice. Mean ± SEM, n=5, *p<0.05, **p<0.01, ***p<0.001, Student's *t*-test.

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