Effects of omega-3 and -6 fatty acids on *Mycobacterium tuberculosis* in macrophages and in mice

Luisa Jordao a, Andreas Lengeling b, Yann Bordat c, Frederic Boudou c, Brigitte Gicquel c, Olivier Neyrolles c,d, Pablo D. Becker e, Carlos A. Guzman e, Gareth Griffithsf, Elsa Anesa, * a

a Molecular Pathogenesis Centre, Unit of Retrovirus and Associated Infections (CPM-URIA), Faculty of Pharmacy, University of Lisbon, Av. Forcas Armadas, 1600-083 Lisbon, Portugal

b The Roslin Institute and Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Roslin, EH25 9RG, UK

c Unité de Génétique Mycobactérienne, Institut Pasteur, Paris, France

d Genetics and Biochemistry of Microorganisms, Centre National de la Recherche Scientifique (CNRS), Paris, France

e Department of Vaccinology, Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany

f EMBL, Postfach 102209, 69117 Heidelberg, Germany

Received 21 April 2008; accepted 6 August 2008

Available online 14 August 2008

Abstract

We recently showed that treatment of macrophages prior to *Mycobacterium tuberculosis* infection with the pro-inflammatory omega-6 lipid, arachidonic acid (AA) enhanced bacterial killing whereas the anti-inflammatory, omega-3 lipid eicosapentaenoic acid (EPA) stimulated bacterial growth. Here we tested if these effects were depending on when lipids were added to macrophages: before or during *Mycobacterium smegmatis* or *M. tuberculosis* infection. Collectively, our data suggested that a high omega-6 diet might be beneficial against mycobacteriosis, while a high omega-3 diet might be detrimental. AA also stimulated TNF-α secretion in *M. tuberculosis*-infected macrophages whereas EPA inhibited this process. AA strongly activated the MAP kinase p38 in uninfected cells but *M. tuberculosis* infected cells blocked the ability of AA to activate p38; AA-dependent killing is therefore independent of p38.

We therefore tested diets enriched in omega-3 and omega-6 lipids on a mouse model of tuberculosis. In contrast to the *in vitro* results, the omega-6 tended to increase survival of *M. tuberculosis* in mice, while omega-3- tended to increase pathogen killing. Overall our results together with those previously reported in the literature suggest that it is almost impossible to predict, at the whole organism level, if a diet enriched in omega-3 or -6 will be beneficial or detrimental to intracellular pathogens.

© 2008 Elsevier Masson SAS. All rights reserved.

Keywords: Lipid signalling; Host–pathogen interaction; Inflammatory response; Phagosome maturation; Intracellular trafficking; MAP kinase p38

1. Introduction

An important part of the life cycle of *Mycobacterium tuberculosis* and related pathogens in infected animals is spent within phagosomes inside macrophages. These pathogens block late fusion events allowing their growth and replication within an arrested early phagosome [1,2]. In contrast, after phagocytic uptake of the non-pathogenic *M. smegmatis*, the phagosomes undergo a complex maturation process involving multiple fusion events with early endosomes and late endocytic organelles. As a consequence of these fusion events, the phagosomes acquire hydrolytic enzymes and the proton ATPase that acidifies the lumen. Collectively, these factors, together with free radicals, contribute to *Mycobacterium smegmatis* killing by macrophages within 24–48 h [3].

In recent studies using latex beads phagosomes we provided evidence for links between the ability of phagosomes...
to assemble actin filaments de novo on their cytoplasmic surface to the late fusion events between phagosomes and late endocytic organelles [4,5]. Indeed, under an increasing set of conditions that stimulate macrophages, the ability of phagosomes to assemble actin correlates well with both their increased fusion with late endocytic organelles and acidification, as well as with an increased ability to kill non-pathogenic and pathogenic mycobacteria. In contrast, conditions that inhibit phagosomal actin assembly are correlated with less fusion and acidification but increased bacterial growth [3,6,7]. This leads us to hypothesize that actin nucleation on membranes is part of the macrophage pro-inflammatory response. The MAP kinase p38 (p38) is a key regulator of this process and many others relevant for the macrophage–phagosome interactions. In recent work we showed that, depending on the stage of infection, some functions regulated by p38 could favour the macrophage or the bacteria [3].

The addition of pro-inflammatory lipids, especially the omega-6 lipid arachidonic acid (AA), to macrophages infected with M. smegmatis or M. tuberculosis was found to have striking effects. This lipid stimulated phagosomal actin assembly, late phagosomal fusion events and mycobacteria killing. In contrast, the anti-inflammatory omega-3 lipid eicosapentaenoic acid (EPA) induced an increase in mycobacterial intracellular growth [6]. These results were exciting because they fitted nicely into a general pattern seen with these classes of lipids in whole organisms. In fact, earlier studies showed that a diet enriched in omega-3 lipids led to significant increases in growth of Salmonella in mice [8] and M. tuberculosis in guinea pigs [9,10].

Collectively, the above data suggested that a diet enriched in omega-6 lipids might be beneficial against mycobacteria, while enrichment in omega-3 might be detrimental. It follows that for patients suffering from tuberculosis and other diseases it is important to detail whether these lipids influence the course of infection, one way or the other.

However, the effects of these lipids on a variety of different pathogens described in the literature, especially at the level of animal models, lead to more sobering conclusions. While these lipids have tremendous effects on many processes related to infections, the results are highly contradictory [11].

Given this situation, we decided to test omega-3 and omega-6 rich diets in mice experimental infection models of M. tuberculosis. As mice were never before tested within this context, we used a Salmonella model in parallel to follow the fate of omegas within mice infected with intracellular pathogens [8,12]. The results obtained were in disagreement with those previously described for salmonella in mice [8] and M. tuberculosis in guinea pigs [9,10].

2. Materials and methods

2.1. Bacterial strain and growth conditions

M. smegmatis mc²155 were grown and maintained as described previously [3,6]. The number of colony forming units (CFU) was evaluated by plating of serial dilutions of bacteria on Middlebrook’s 7H10. For M. tuberculosis H37rv culture media were supplemented with 10% oleate albumin dextrose catalase (OADC -Difco).

The virulent Salmonella enterica serovar Typhimurium (ATCC 14028) was routinely grown at 37 °C in brain heart infusion (BHI) broth or agar (Difco). For challenge studies, bacteria were grown at 37 °C with gentle shaking (50 rpm) until they reach an OD₆₀₀nm of 0.8. The number of viable bacteria was evaluated by plating serially diluted samples on BHI agar plates.

2.2. Animal experiments

2.2.1. Animals and diets

Animals and diets: For Salmonella infection, female BALB/c (H-2d) mice (10 weeks old) were purchased from Harlan–Winkelmann GmbH (Borchen, Germany). Animals received a fat-free diet (ssniff® EF R/M without fat addition from ssniff Spezialdiäten GmbH) for 1 week, then they were divided into three groups. Each group received one of the following diets: (i) 3.5% of soy oil (normal fat content: control), (ii) 10% of sunflower oil (omega-6, linoleic acid content 7.41%), and (iii) 10% of menhaden oil (omega-3, EPA content 1.30% and DHA content 0.85%). Food pellets were changed daily to preserve lipid content and properties.

For M. tuberculosis infection, 6–8-weeks-old female BALB/c mice were fed with the fat-free diet described above, supplemented or not with 10% safflower oil (omega-6, linoleic acid content 7.50%), or 10% Ropufa (omega-3, EPA content 1.5% and DHA 1.1%). Special diets started 2 weeks before infection and were maintained during the course of infection.

2.2.2. Challenge experiments

After 5 weeks on the fat-specific diets, mice were gently fed with 10⁶ CFU of Salmonella. Bacterial invasion, survival and in vivo dissemination were evaluated. Three mice from each group were euthanized on days 3 and 7. Spleen, liver, Peyer’s patches (PP) and mesenteric lymph nodes (MLN) were removed, weighed and homogenized in sterile PBS. The number of viable bacteria present in the organs was determined by plating on BHI agar plates. The results were expressed as number of CFU/g of tissue.

For M. tuberculosis experiments, after 2 weeks receiving fat specific diets, mice were infected intranasally with 10⁵ CFU of M. tuberculosis. As mice were never before tested within this context, we used a Salmonella model in parallel to follow the fate of omegas within mice infected with intracellular pathogens [8,12]. The results obtained were in disagreement with those previously described for salmonella in mice [8] and M. tuberculosis in guinea pigs [9,10].

2.3. Macrophage infection and survival experiments

The mouse macrophage cell line J774A.1 was cultured and infected with M. tuberculosis and M. smegmatis as described previously [3,6]. In all experiments lipid solutions in ethanol (1 mg/ml) were extemporaneously prepared and added to culture medium in optimal concentrations. These concentrations were selected based on the effects on intracellular
mycobacteria survival. The minimum concentration effective on bacteria killing/survival and lacking toxic effects on macrophages, were the following: 125 \( \mu M \) AA and 15 \( \mu M \) EPA.

Solvent without lipid was routinely tested (Mock control) and referred as control in all figures. In experiments using the post-treatment with lipids these were added after washing out extra-cellular bacteria at the end of the bacterial uptake period. Lipids were left in contact with infected macrophages afterwards. In pre-treatment experiments the lipids were added everyday for 3 days before the start of the infection without additional lipids added afterwards.

2.4. TNF-\( \alpha \) assay

TNF-\( \alpha \) was assessed in culture supernatants by ELISA (Quantitine TNF-\( \alpha \) kit, R&D).

2.5. p38 immunoblot analysis

When needed lipid treatment and/or infection were performed as described in Section 2.3. Total cellular extracts were prepared as described previously [3]. Equal amounts of protein were loaded on a 12% SDS—PAGE, transferred to nitrocellulose membrane and probed with a rabbit antiphospho-p38 and anti total p38 (Cell Signalling Technology). Enhanced chemi-luminescence (Pierce Biotechnology) was used to visualize antibody binding. Total p38 was used as a loading control.

2.6. Statistics and data presentation

All values are reported as means \( \pm SD \) of three independent experiments. The statistical significance of the differences observed in bacterial loads was analyzed by the Student’s \( t \)-test, whereas those from the mice survival studies were analyzed using the log rank test. Differences were considered significant at \( P < 0.05 \) (*) or \( P < 0.01 \) (**).

3. Results

3.1. Effect of EPA and AA on M. tuberculosis intracellular growth

We first tested different concentrations of an omega-3 (EPA) and an omega-6 lipid (AA) on J774 macrophages infected with M. tuberculosis H37Rv. Fig. 1A shows the results obtained with the different concentrations of EPA (5, 15 and 150 \( \mu M \)) and AA (62.5, 125 and 250 \( \mu M \)) on the intracellular survival of M. tuberculosis H37Rv on J774 macrophages. Based on these and earlier results we selected the lowest concentration of lipids that enhanced growth (EPA; 15 \( \mu M \)) or killing (AA; 125 \( \mu M \)) without affecting macrophage viability [6].

We next asked if the effect of the lipids was dependent on the period of contact with the infected cultures. Lipids were added at their optimal concentrations to macrophages for 3 days before infection (pre-treatment) or by daily additions after infection (post-treatment). Lipid treatment had no effect on the rate of bacterial uptake (Fig. 1A–C).

In accordance with our previous data [6] M. tuberculosis survival within macrophages was enhanced by EPA and decreased by AA addition (Fig. 1A). It was striking that pre-treatment with AA was just as effective as post-treatment in enhancing bacterial killing (Fig. 1B,C). For EPA the main difference between the two treatments was that whereas pre-treatment led to a continual growth of M. tuberculosis until day 7, the post-treatment induced significantly more growth at day 3 than at day 7 (Fig. 1B,C).

Recently we characterized, in great detail, the infection of J774 macrophages by M. smegmatis, a fast growing, non-pathogenic mycobacteria [3]. In this model we observed alternate pro- and anti-inflammatory phases so we asked if the addition of lipids would have different effects if supplemented only during each specific pro or anti-inflammatory phase. The rationale is that a pro-inflammatory lipid might accentuate the effects of a pro-inflammatory phase (increasing killing), while it might reduce the rate of growth seen in the anti-inflammatory phases (and vice-versa for an anti-inflammatory lipid).

We added these lipids, at concentration optimized for M. tuberculosis, at different time windows starting 1 h before infection, with the last period being from 16 to 24 h, a period when the vast majority of bacteria are normally killed [3]. We estimated CFU of bacteria isolated from cells after 24 h infection (more or less one generation time of M. tuberculosis). The overall trend observed for M. tuberculosis, with EPA enhancing survival and AA enhancing killing, was also observed for M. smegmatis (Fig. 2). A striking effect on M. smegmatis growth was seen when EPA was added from –1 to 4 h contrasting with the absence of effect for additions between zero and 4 h (Fig. 2). This argues that a 1 h ‘priming’ of macrophages with EPA before infection enhances the anti-inflammatory state of these cells for the next 24 h. However, when AA was added –1–4 h the opposite effect (killing) was not observed. Nevertheless, addition of this lipid, in 4 h time windows, between 4 and 24 h led to a statistically significant increase in killing (Fig. 2).

The overall trend of the results observed was similar for both mycobacteria species tested. Since M. tuberculosis infection is one of the most relevant infectious diseases we decided to investigate further the molecular mechanisms involved in this model.

3.2. Lipids and TNF-\( \alpha \) secretion

In macrophages infected with pathogenic mycobacteria TNF-\( \alpha \) is known to be an important cytokine whose extra-cellular levels correlate strongly with mycobacteria killing, both in in vitro and in animal models [13,14]. In in vitro macrophage infection the secreted TNF-\( \alpha \) has the potential to activate the TNF-\( \alpha \) receptor and to trigger an autocrine signalling cascade. We therefore asked whether EPA or AA had any effect on the secretion of TNF-\( \alpha \) in macrophages infected with M. tuberculosis. In uninfected cells addition of
both lipids did not affect the basal level of TNF-α secretion (200 pmol/ml; Fig. 3). Both pre- and post-treatment with AA increased secretion of this cytokine by M. tuberculosis infected macrophages. In contrast pre-treatment with EPA reduced TNF-α secretion whereas post-treatment had no effect (Fig. 3). Collectively these results support the notion that the pro-inflammatory lipid AA tend to increase TNF-α secretion whereas the anti-inflammatory lipid EPA had the opposite effect.

3.3. Lipids and p38 MAP kinase activity

Pro-inflammatory TNF-α downstream signalling events could lead to MAP kinase p38 activation, which is a crucial player in mycobacterial infections [3,15,16]. Therefore we investigated the effects of the lipids on p38 activation, as monitored by its specific phosphorylation, using an antibody against phospho-p38. In uninfected cells, without lipid treatment or treated with EPA, p38 activation was not detected over 5 days. In contrast AA was able to induce p38 activation after 3 h of contact. This signal persisted until 1 day and even increased until day 5 (Fig. 4A).

When cultures infected with M. tuberculosis were treated with EPA or AA, in a post-treatment fashion, p38 phosphorylation was absent up to day 5 (Fig. 4B). Collectively, these data argue that AA-induced killing of M. tuberculosis is independent of p38.

3.4. Effects of omega-3 and omega-6 enriched diets on the infection of mice with Mycobacterium tuberculosis

We next tested the effect of omega-3 and omega-6-enriched diets on the survival of M. tuberculosis in mice. Animals were divided into three groups and fed with different diets: standard diet (control) and test diets enriched in omega-3 or omega-6 lipids, during 2 weeks prior to infection by aerosol.

The results of CFU recovered from lungs and spleen after different periods of time are presented in Fig. 5A. The omega-3 diet led to a slight, but statistically significant, reduction in mycobacterial loads in the lungs and spleen, both at 21 and
63 days after inoculation (Fig. 5A). This result suggests that this diet had a small anti-mycobacterial effect in vivo rather than being conducive to pathogen survival, as we had predicted. The omega-6 enriched diet tended towards opposite effects in spleens and, rather than inducing killing, tends to help bacteria survival.

Since the mouse model of tuberculosis was never tested for the effects of diets enriched in omegas we assessed the salmonella model, in parallel [8]. This intracellular pathogen, like M. tuberculosis, is able to arrest phagosome maturation in macrophages [17]. In addition, an omega-3 enriched diet was described by Chang and co-workers to lead to a significant survival of Salmonella in mice (in agreement with our in vitro results with M. tuberculosis) [8]. Fig. 5B shows the number of bacteria recovered from PP, MLN, spleen and liver at different time intervals. On day 3 post-infection, similar numbers of bacteria were recovered in PP and MLN from mice under all treatment conditions (Fig. 5B), arguing that bacterial capacity to infect, invade and survive within the host was not affected by any of the diets. However, bacterial loads recovered from the spleen of animals receiving the omega-6 diet were significantly increased. This pattern was even more striking on day 7 post-infection (Fig. 5B), since in all tested organs the number of bacteria was significantly higher than in the groups receiving the standard diet.

Fig. 2. Persistence of M. smegmatis in cultures of J774 macrophages supplemented with AA or EPA during distinct phases of intracellular killing cycles. Effect of 125 μM AA or 15 μM EPA on intracellular killing of M. smegmatis. Lipids were added in specific time windows to macrophages as represented and bacteria were recovered for CFU plating 24 h post-infection. The difference between the experimental conditions tested was considered statistically significant at P < 0.05 (*). The upper bar rectangles indicate the period of contact with the lipid and the arrow the time in which bacteria were recovered for CFU counting.

Fig. 3. Effects of lipids on TNF-α pro inflammatory response in J774 cell culture infected with M. tuberculosis. The supernatants of the infected cultures either treated before infection (pre-treatment) or post bacteria uptake with lipids (post-treatment) were assessed for TNF-α production. There is no effect of lipids observed in uninfected cells (Lipid-control/cells). The difference between the experimental conditions tested was considered statistically significant at P < 0.05 (*).

Fig. 4. Lipid-induced phosphorylation of p38 in J774 macrophages during M. tuberculosis infection. (A) Western blot for phospho-p38 and total p38 for uninfected macrophage not treated (control) or treated with lipids (+AA, +EPA) up to 5 days. (B) Western blot for phospho-p38 and total p38 for macrophages infected with M. tuberculosis not treated (control) or treated with lipids (+AA, +EPA) up to 5 days.
These results suggest that animals receiving the omega-6 diet were less efficient in controlling bacterial spread and dissemination. The omega-3 diet, initially, led to fewer bacterial numbers. However, in the long term none of the diets was able to confer full protection against a lethal challenge. Although mice receiving control and omega-3 diets survived longer than those on the omega-6 diet, the differences were not statistically significant. But overall in the tuberculosis and salmonellosis mouse models of infection the omega-3 enriched diets tended to induce bacterial killing (statistically significant for \textit{M. tuberculosis}) while the omega-6 supplementation helped bacterial survival (statistically significant for salmonella).

4. Discussion

The striking and opposite effect of AA (omega-6) and EPA (omega-3) on intracellular persistence of non-pathogenic and pathogenic mycobacteria were previously reported using lipid treatment before infection [6]. We asked whether lipid supplementation before or after infection could induce different outcomes. Our data showed no distinct effects: overall EPA significantly enhanced mycobacteria growth while AA led to a strong bactericidal effect. The ability of these lipids to induce bacterial growth or killing could be correlated with their effects on the secretion of TNF-$\alpha$. EPA inhibits TNF-$\alpha$ secretion by \textit{M. tuberculosis}-infected macrophages whereas AA stimulated this process. The latter pattern was also reflected in the ability of AA, but not EPA, to strongly activate the MAP kinase p38 in uninfected macrophages. However, p38 was not activated in \textit{M. tuberculosis}-infected macrophages, even when AA was added. Thus, the ability of AA to facilitate killing of \textit{M. tuberculosis} is independent of p38 activity.

The availability of human and animal data showing the same effect with \textit{Salmonella} and \textit{M. tuberculosis} made it an attractive hypothesis that EPA, used as a human nutritional supplement, should be avoided in infected people. Especially during latent tuberculosis, an increased risk of disease reactivation may be observed. This also made the alternative hypothesis, that pro-inflammatory lipids might have therapeutic effects against tuberculosis and related diseases, more interesting from a medical perspective.

We tested in our established models similar conditions to those used by Chang and co-workers for which omega-3 supplementation enhanced salmonella growth in mice [8]. Since we have to set up the system to assess the effects of omega lipids on a mouse model of tuberculosis, we used as reference a model already described in guinea pigs [9,10]. Therefore for both pathogens BALB/c mice were used and challenged with a lethal dose of each bacteria using their usual route of entry (oral for salmonella and aerosols for \textit{M. tuberculosis}). An omega-3 or -6 enriched diet was established during 2-4 weeks before infection in order to allow cell membrane enrichment in a particular lipid [8,11,18]. Lipid diets were maintained during the course of infection. These are the crucial parameters established in animal models of infectious diseases described in the literature [8,11,19,20]. Because the animal strain used was reported to play an important role we adopted BALB/c female mice for both models of infection. Moreover, mouse macrophages from a BALB/c background
were used in in vitro experiments, making this mouse strain the animal of choice for correlating in vitro and in vivo results.

Feeding EPA and DHA has been shown to modulate specific functions of innate and acquired immunity. In general, high feeding levels (>10% of total fat) of n-3 PUFA (compared with diets high in n-6 PUFA) to healthy animals or human subjects results in suppression of the ability of lymphocytes to respond to mitogen stimulation, NK cell activity and delayed-type hypersensitivity reactions [21]. Suppression of these functions has been demonstrated by feeding animals from 1% w/w purified EPA or DHA (docosahexaenoic acid) [22] up to 20% w/w fish oil (approximately 3.5% w/w EPA-DHA) [23]. Moreover, we established enriched diets up to 10% w/w omega-3 or omega-6 for both models of infection [9]. Feeding long-chain n-3 PUFA was shown to significantly decrease the production of interleukin (IL)-1, IL-6, and TNF-α by peripheral blood mononuclear cells in humans and by peritoneal macrophages in animals after mitogen stimulation [24]. However, there is some discrepancy with respect to TNF-α production in animals, because some studies document increases in TNF-α production after stimulation when animals were fed high fat diets (20% fat wt/wt) rich in fish oil for 4 weeks [25].

Conversely, a number of studies have shown that feeding more moderate amounts of n-3 PUFA (i.e., fed at <10% of fat to animals and <1 g EPA + DHA/day to humans) is not immunosuppressive [26], and can even enhance immune functions such as lymphocyte proliferation/activation, NK cell activity, macrophage activation [27], and IL-1, IL-2, and TNF-α production after mitogen stimulation [28]. Due to experimental and economical constraints our data can only be considered as preliminary since only a limited set of conditions was tested. Nevertheless this may explain our results in mice infected by Salmonella or by Mycobacterium tuberculosis with a tendency of omega-3 to have no effect or to kill pathogens rather than increasing survival.

TNF-α together with mediators of acute inflammation such as IL-1β, PGE2 and leukotriene B4 (LTB4) are produced in response to pathogens. The last two mediators are final products of AA conversion by cyclo-oxygenase and 5-lipoxygenase (5-LO), respectively. Lipoxins represent another class of 5-LO-derived eicosanoids, but in contrast to LTs, these mediators possess anti-inflammatory properties [29]. Therefore, in animals downstream breakdown products of the omega-6 added to the diet could play opposite and more complex effects, thus reflecting the disparity of results found on the outcome of the infection.

Given that several pro- and anti-inflammatory mediators could result from the same dietary lipid during the course of infectious diseases such as tuberculosis, we suggest that further testing would be prudent. Collectively our data argue against the idea of considering a simple recommended lipid-based diet against mycobacteria and other intracellular pathogens.

Acknowledgements

We thank Cécilia Rodrigues, Maximilliano Gutierrez, Luis Mayorga and Sabrina Marion for their constructive support and discussion. This work was financed by FCT with co-participation of FEDER (POCI/BLA-BCM/55327/2004 and SRHR/BD/14284/2003).

References


