The effect of replacement of dietary fat by palm oil on in vitro cytokine release

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In the present study the effect of replacement of dietary fat by palm oil in the normal Western diet on the in vitro release of the inflammatory cytokines tumour necrosis factor (TNF), interleukin (IL)-6 and IL-8 was examined. A maximal replacement of 700 g/kg dietary fat was achieved for thirty-eight male volunteers who consumed either a palm-oil diet or a control diet in a double-blind, cross-over study with 6-week experimental periods, and 3-week run-in and wash-out periods. At the end of both experimental periods, whole blood was stimulated in vitro with 0.02 (sub-optimal), or 10 ng lipopolysaccharide (LPS)/ml (maximal), whereafter TNF, IL-6, and IL-8 concentrations in the culture supernatant fraction were measured using specific enzyme-linked immunosorbent assays (ELISA). Mean cytokine production with sub-optimal, or maximal LPS stimulation of peripheral whole blood was similar for both the palm oil, and the control group. The relative TNF response, however, was reduced by replacement of dietary fat with palm oil. Separate analysis of the data from the first and second experimental periods strongly suggested that the residual effect of the palm-oil diet on the relative TNF response was longer than 9 weeks. Cytokine homeostasis determines the course of the inflammatory response and the progression of atherosclerosis. The effect of palm-oil consumption on the proneness of the peripheral blood cells to produce TNF may, therefore, alter the prevalence of these common diseases.

Cytokine production: Palm-oil diet: Tumour necrosis factor

Dietary fatty acid composition affects the inflammatory response and the cardiovascular risk profiles in animal models (Freed et al. 1989; Lefkowith et al. 1990b) and in man (Kremer et al. 1987). Dietary supplementation with fish oil (the main source of n-3 polyunsaturated fatty acid) leads to improvements in patients with psoriasis (Bittiner et al. 1988), rheumatoid arthritis (Kremer et al. 1987) and asthma (Payan et al. 1986). Epidemiological studies have shown that a high intake of fish oil correlates with a low incidence of cardiovascular disease (Dyerberg et al. 1975; Bang et al. 1976) and immune disorder-related diseases, such as Type I diabetes mellitus, psoriasis and asthma (Kromann & Green, 1980).

The mechanisms underlying the protective effect of the consumption of fatty acids such as those in fish oil is being investigated intensively. Recently, the influence of fish oil consumption on the inflammatory response of mononuclear phagocytes, characterized by the production of cytokines, was reported (Endres et al. 1989b). Cytokines are hormone-like molecules with an immunoregulatory function which are produced by cells of the immune

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system. The production of the cytokines interleukin (IL)-1β, IL-1α, and tumour necrosis factor (TNF) by in vitro stimulated peripheral blood mononuclear cells was shown to be suppressed during nutritional supplementation with n-3 polyunsaturated fatty acids (Endres et al. 1989a). The anti-inflammatory effect and the beneficial effect on progression of cardiovascular disease of fish oil consumption may be mediated by its inhibitory effect on the production of cytokines by mononuclear phagocytes, since the products of these immune cells sustain and enhance the process of atherogenesis (Ross, 1986), and have a regulatory function in the immune response (Kunkel et al. 1989). The activity of monocytes and their capacity to produce cytokines are, therefore, considered to be important determinants for the course of both cardiovascular and immune disorder-related diseases.

In the present study the effect of replacement of dietary fat with palm oil (a vegetable oil containing approximately equal proportions of saturated and unsaturated fatty acids, but free from cholesterol and trans-fatty acids) on the in vitro production of the cytokines TNF, IL-6, and IL-8 was investigated. The study was performed as part of a trial that investigated the influence of palm-oil consumption on some cardiovascular risk profiles of healthy males (Sundram et al. 1992).

Mean production of the inflammatory cytokines TNF, IL-6, and IL-8 after stimulation of whole blood with 0.02, or 10 ng lipopolysaccharide (LPS)/ml remained unaffected by palm-oil replacement of dietary fat. However, strong evidence was obtained that the proneness of the peripheral blood cells to produce TNF was reduced by palm-oil replacement of dietary fat.

**MATERIALS AND METHODS**

*Subjects and study design*

Maximum replacement of dietary fat with palm oil was evaluated for its effect on the in vitro cytokine secretion by whole blood stimulation of thirty-eight healthy male volunteers, using a double-blind cross-over design. The research protocol was approved by the Medical Ethical Committee of the University of Limburg, Maastricht, The Netherlands. The screening, selection and randomization criteria of the volunteers have been described in detail previously (Sundram et al. 1992). In short, forty non-obese, non-smoking, and healthy (no medication, absence of glucose and protein in urine) male volunteers entered the study after a written informed consent (average age 36 years, range 19–45 years). Two subjects dropped out of the study, one because of job commitments, and the other due to medication prescribed by his physician. In a series of dietary products contributing a high fat intake in the Dutch diet, the normal fat component was replaced by palm oil. A control series of the same products was prepared, which contained fats and oils, reflecting fat in the normal Dutch diet, but excluding palm oil. The study was designed as a double-blind cross-over trial, consisting of two periods of 6 weeks each, preceded by a run-in period of 3 weeks and interrupted by a wash-out period of 3 weeks. During the run-in period and the wash-out period, all volunteers consumed the control products. The experimental group, consisting of eighteen subjects, was provided with the palm-oil-substituted products during the experimental period of 6 weeks, and the control group received the control products. In the second experimental period volunteers who consumed the palm-oil products in the first period consumed the control diet, and vice versa. The volunteers were instructed to maintain their normal dietary habits during the experiment.

Through a continuous computerized monitoring system, repeated dietary histories and duplicate portion analyses, it was estimated that up to 700 g/kg substitution of the dietary fat with palm oil was achieved. No differences were observed between the control group (CT) and the palm-oil group (PO) concerning energy intake (CT 14.4 (SEM 0.48), PO 14.6...
PALM OIL AFFECTS IN VITRO CYTOKINE RELEASE

(SEM 0.44) MJ/d; or fat energy intake (CT 41 (SEM 0.69), PO 41 (SEM 0.78) %; both estimated on basis of dietary history). During palm-oil substitution the intake of energy from saturated fatty acids was higher in comparison with the control diet (polyunsaturated:saturated (P:S) fatty acids CT 0.43 (SEM 0.01), PO 0.40 (SEM 0.01); P = 0.001).

The production of TNF, IL-6 and IL-8 by LPS-stimulated whole blood in vitro was assessed at the end of each experimental period. From each volunteer, 5 ml venous blood was sampled in sterile pyrogen-free syringes containing 50 IU thromboliquine (Organon Teknika, Boxtel, The Netherlands). For optimization in the laboratory, blood sampling was performed on eight separate days (five volunteers each day). Within each day, volunteers were subdivided between the two groups in such a manner that an equal number of volunteers was assigned to the two dietary groups by the end of each second day. The volunteers entered the study in a staggered way, assuring that the experimental periods were exactly the same for all participants.

In vitro stimulation of whole blood
The heparinized venous blood was divided over nine tubes (0.5 ml blood/tube). The tubes were centrifuged, the plasma fraction was discarded and the total cell fraction was resuspended in 1 ml RPMI 1640 (Gibco, Paisley, UK) supplemented with 100 IU Penicillin G/ml and 100 μg Streptomycin/ml. The cell suspension was transferred to a twenty-four-macrowell plate (Costar, Cambridge, MA, USA) and stimulated in triplicate with 0, 0.02, or 10 ng LPS Escherichia coli O55:B5 (Sigma, St Louis, MO, USA)/ml. After 4 h of incubation at 37°, 50 ml carbon dioxide/l in humidified air, the plates were centrifuged, culture supernatant fraction was harvested and stored at –20°.

Determination of TNF
The TNF concentration in the culture supernatant fraction of whole blood after 4 h of stimulation was determined using an enzyme-linked immunosorbent assay (ELISA) specific for TNFα. Details on the specificity and sensitivity have been reported elsewhere (Engelberts et al. 1991a). TNF concentrations in all samples were determined within 2 weeks, to avoid storage artifacts.

Determination of IL-6
The culture supernatant fraction IL-6 concentration was determined using a sandwich ELISA for human IL-6. A ninety-six-well immuno maxisorp plate (Nunc, Roskilde, Denmark) was coated overnight at 4° with murine monoclonal antibody 8, specific for human IL-6 (Aarden et al. 1987). The standard titration curve was obtained by making a serial dilution of a known quantity of a human recombinant IL-6 sample. Test samples, diluted in RPMI 1640, were added to the plates and incubated during 2 h at room temperature. Next, the plates were incubated with a polyclonal rabbit anti-human IL-6 antiserum, followed by a peroxidase-conjugated goat anti-rabbit-IgG (Jackson, Westgrove, PA, USA). Substrate (o-phenyldiamine; Sigma) was added and absorbance was measured.

Determination of IL-8
A specific ELISA was used to determine the IL-8 concentration (Ceska et al. 1989). A ninety-six-well immuno maxisorp plate (Nunc) was coated overnight at 4° with a polyclonal goat anti-human IL-8 antiserum (5 μg/ml). A standard titration curve was obtained by making a serial dilution of a known quantity of a human recombinant sample. Test samples were added to the plates and incubated during 2 h at 37°. Next, the plates were incubated with a biotin-conjugated polyclonal goat anti-human IL-8 (2.5 μg/ml), followed by...
peroxidase-conjugated avidin (Bio-Rad, Richmond, CA, USA). Substrate (α-phenyldiamine) was added and absorbance was read.

Statistics
Results are presented as means with their standard errors, or medians where indicated. The data were calculated for the end of the PO and CT periods. Testing of the PO v. CT effect was done using the two-sample t test on the data at the end of the first period. Since little is known of the retention time of PO effect on the in vitro cytokine release, second-period data were used to obtain information on the residual palm-oil effect after 9 weeks. The residual PO effect was tested by the interaction test for cross-over trials (Armitage & Berry, 1987). For variables where the distribution seemed to be non-normal, Mann–Whitney tests were applied as well. Two-sided values of $P < 0.05$ were considered to be significant.

To investigate whether the maximal TNF-production correlated with the maximal IL-6 or IL-8 production, the Spearman correlation coefficients for these values were calculated for the control group at the end of the first experimental period.

RESULTS AND DISCUSSION
The in vitro production of TNF, IL-6 and IL-8 by LPS-stimulated (10, 0.02 ng/ml, and non-stimulated) whole blood was assessed at the end of the PO and CT periods. LPS-stimulation was performed on whole blood, in order to maintain the monocytes in their physiological environment (Desch et al. 1989).

The in vitro cytokine production was specified by two variables. First, by the mean cytokine production, that is the mean of the cytokine concentrations for one experimental group in the supernatant fraction of whole blood after 4 h of stimulation with 0.02 (sub-optimal), or 10 (maximal) ng LPS/ml. The amount of LPS required for maximal effect varies greatly, and is dependent on several factors such as the presence of serum lipoproteins (Harris et al. 1990) or the recently described bactericidal/permeability-increasing protein (Marra et al. 1990), which both inhibit the cytokine response on LPS-stimulation. In contrast, the serum component LBP is essential for LPS-stimulation of macrophages by means of surface antigen CD14 (Tobias et al. 1988). Moreover, increase in polyunsaturated fatty acid composition of the cellular membrane enhances susceptibility to LPS (Stark & Jackson, 1990). Stimulation with 10 ng LPS/ml, therefore, does not induce maximal cytokine production in every in vitro model for leukocyte stimulation. However, in the experimental setting used, 10 ng LPS/ml induced maximal cytokine production in 95% of the tested samples.

Second, the relative response (%) was used to describe in vitro cytokine production. Relative cytokine response was calculated as follows:

$$\frac{\text{cytokine concentration after stimulus with 0.02 ng LPS/ml}}{\text{cytokine concentration after stimulus with 10 ng LPS/ml}} \times 100\%.$$

The relative response compares the sub-optimal cytokine production with the maximal cytokine production for each whole blood stimulation separately. Relative cytokine response is independent of leukocyte count or differentiation, and is a measure for the proneness of peripheral blood cells to produce cytokines on LPS stimulation.

The cytokine production was below detection limits when no LPS stimulus was added (values not shown). Mean TNF, IL-6, and IL-8 production after stimulation of whole blood in vitro with 0.02, or 10 ng LPS/ml, at the end of the first and of the second
Table 1. Cytokine production (ng/ml) by lipopolysaccharide (LPS)-stimulated whole blood in vitro in subjects consuming a control diet or a diet in which palm oil replaced a maximum of 700 g/kg dietary fat*

(At the end of the first and of the second experimental periods, cytokine concentration was measured in the culture supernatant fraction of whole blood. Whole blood was stimulated during 4 h \textit{in vitro} with 0.02 (sub-optimal), or 10 (maximal) ng LPS/ml. Values are with their standard errors for no. of subjects shown in parentheses)

<table>
<thead>
<tr>
<th></th>
<th>First experimental period</th>
<th>Second experimental period</th>
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<tr>
<td></td>
<td>Palm-oil diet (18)</td>
<td>Control (20)</td>
</tr>
<tr>
<td></td>
<td>Control (20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>TNF: Max</td>
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</tr>
<tr>
<td>Sub-optimal</td>
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<td>0.27</td>
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<td>IL-6: Max</td>
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<tr>
<td>Sub-optimal</td>
<td>0.6</td>
<td>0.06</td>
</tr>
<tr>
<td>IL-8: Max</td>
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<td>0.14</td>
</tr>
<tr>
<td>Sub-optimal</td>
<td>1.4</td>
<td>0.09</td>
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NS, not significant; TNF, tumour necrosis factor; IL, interleukin.
* For details of diets and procedures, see pp. 160-162.

Table 2. \textit{Intra-individual correlation for maximal cytokine production in twenty subjects consuming a control diet}†

(The intra-individual correlation for maximal cytokine production was calculated for the control group at the end of the first experimental period (Spearman correlation coefficients are given))

<table>
<thead>
<tr>
<th></th>
<th>Maximal TNF</th>
<th>Maximal IL-6</th>
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<tbody>
<tr>
<td>Maximal IL-6</td>
<td>0.488*</td>
<td>1</td>
</tr>
<tr>
<td>Maximal IL-8</td>
<td>0.618**</td>
<td>0.780***</td>
</tr>
</tbody>
</table>

IL, interleukin; TNF, tumour necrosis factor.
* \( P = 0.03 \), ** \( P = 0.004 \), *** \( P = 0.0001 \)
† For details of diet and procedures, see pp. 160-162.

experimental period, were equal during the PO diet, compared with the CT diet (Table 1). A period effect was observed for mean IL-6 and TNF release (Table 1). We have no explanation for this. As appears from different influences of pentoxyfylline (Zabel \textit{et al.} 1989) and platelet activating factor (PAF)-antagonists (Engelberts \textit{et al.} 1991\textit{b}) on the production of TNF and IL-6, we know that differences in regulation of the release of these cytokines exist. However, detailed knowledge on the regulation of the release of various cytokines is missing until now.

The \textit{intra-individual} correlations between the maximal TNF \textit{v.} IL-6 production, maximal TNF \textit{v.} IL-8 production, and maximal IL-6 \textit{v.} IL-8 production were determined. In order to prevent a possible influence of palm oil on the correlation variables, only the CT group data at the end of the first experimental period were considered. The findings presented in Table 2 demonstrate that a high maximal \textit{in vitro} production of TNF predicts a high maximal IL-6 and IL-8 production, which suggests that the quality which determines a high maximal TNF production is similar to that which allows a high IL-6, or IL-8 production.
The relative cytokine responses varied largely between individuals (Fig. 1; range 20–200 % for TNF at the end of the first experimental period). The amount of LPS needed to induce a given part of the maximal TNF production was an unstable entity. At the end of the first experimental period the relative TNF response was reduced for the PO group compared with the CT group (\( P = 0.02 \), \( t \) test; \( P = 0.04 \), Mann–Whitney test; Table 3). These findings show that in our experimental setting the peripheral blood cells of the PO-fed volunteers required a stronger LPS-stimulus to reach a given TNF production. The relative responses for IL-6 and IL-8 were not influenced by palm-oil consumption (Table 3).

The functional changes induced by manipulation of fatty acid consumption are prone to persist for a longer time. Changes in cytokine production are observed as late as 10 weeks after discontinuation of \( n-3 \) fatty acid supplementation (Endres et al. 1989b). Also, the biochemical changes associated with \( n-3 \) fatty acid supplementation are sustained (Kremer et al. 1987). This phenomenon implies that the usual analysis for cross-over trials (Armitage & Berry, 1987) may not be applicable here. The difference in the mean relative TNF response between the PO group and the CT group, observed at the end of the first experimental period, had disappeared at the end of the second period when the diets were crossed for each experimental group (Fig. 1). Although the residual palm-oil effect on the relative TNF response did not reach significance at \( P < 0.05 \) (\( P = 0.06 \), \( t \) test), a persistence of the PO diet-induced changes for at least 9 weeks is suggested by these findings.

The mechanism underlying the influence of dietary fatty acid manipulation on cytokine secretion is unknown. The modulating effect of consumption of \( n-3 \) polyunsaturated fatty acids on eicosanoid metabolism (Lee et al. 1985; Lefkowith et al. 1990a) is proposed as an explanation for the suppression of the IL-1\( \alpha \), IL-1\( \beta \), and TNF synthesis during fish-oil consumption (Endres et al. 1989a,b). Palm oil is not rich in polyunsaturated fatty acids. It is, therefore, unlikely that the reduction of the relative TNF response in the present experiment was mediated by specific influences of polyunsaturated fatty acids on the regulation of cytokine production.

However, the proneness of mononuclear phagocytes to produce TNF might be altered via the influence of vitamin E on the regulation of lipid cyclo-oxygenation, and peroxidation product generation. Several investigators have shown the influence of vitamin E consumption on the immune response (Harman & Miller, 1986; Meydani et al. 1986). The modulation of the eicosanoid metabolism by vitamin E consumption (Meydani et al. 1986) is considered to be central to the effects on the immune response and atherogenesis, whereas it is known that arachidonic acid metabolites have a potent regulatory effect on the TNF production (Scales et al. 1989; Waymack et al. 1990). The physiological activity of vitamin E is generally attributed to \( \alpha \)-tocopherol which is present in most edible oils, including fish oil and palm oil. The vitamin E constituent of palm oil, however, is a mixture of tocopherols and tocotrienols (Sundram et al. 1990). There is mounting evidence that palm oil tocotrienols have different biological activities compared with \( \alpha \)-tocopherol (Komiyama et al. 1989). Whether these vitamin E isomers were responsible for a reduced relative TNF response as a consequence of the experimental PO diet, remains to be elucidated.

The activity of monocytes and their capacity to produce cytokines are important determinants for the course of the inflammatory response and the progression of atherosclerosis. The products of these immune cells sustain and enhance the process of atherogenesis (Ross, 1986), and have a regulatory function in the chronic immune response (Kunkel et al. 1989). Furthermore, recent evidence indicates that TNF inhibits the development of acute autoimmune diabetes (Satoh et al. 1990). Palm oil is a potential candidate to constitute a substantial part of the fat content of the Western diet. Minor palm
Table 3. Relative cytokine response (%) by whole blood from subjects consuming a control diet or a diet in which palm oil replaced a maximum of 700 g/kg dietary fat† to a stimulus with lipo-polysaccharide (LPS) in vitro

(The response was calculated as the percentage of the maximal cytokine production that was reached on a sub-optimal stimulation of whole blood in vitro with LPS at the end of the first experimental period. Values are means with their standard errors for no. of subjects shown; medians are given in parentheses)

<table>
<thead>
<tr>
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<th>Palm-oil diet (n 18)</th>
<th>Control diet (n 20)</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>TNF (%)</td>
<td>53</td>
<td>4</td>
</tr>
<tr>
<td>(54)</td>
<td></td>
<td></td>
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<tr>
<td>IL-6 (%)</td>
<td>51</td>
<td>5</td>
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<tr>
<td>(50)</td>
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<td></td>
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<tr>
<td>IL-8 (%)</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>(77)</td>
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</table>

* P = 0.02 (t test); P = 0.04, (Mann-Whitney test).
† For details of diets and procedures, see pp. 160–162.

Fig. 1. Interindividual distribution for the relative tumor necrosis factor (TNF) response at the end of the first and of the second experimental periods. Whole blood from thirty-eight healthy individuals, who consumed either a control diet or a palm-oil diet by a cross-over protocol, was stimulated during 4 h in vitro with 0.02, and 10 ng lipopolysaccharide (LPS)/ml. The relative TNF response (%) was calculated as the portion of the maximal TNF response (at 10 ng LPS/ml) that was reached at a sub-optimal stimulus (0.02 ng LPS/ml). The results are the mean of triplicate measurements with the medians represented by horizontal lines. For details of diets and procedures, see pp. 160–162.

Oil-induced changes in the setpoint of cytokine production may, therefore, have important consequences for the prevalence and the course of common diseases such as immune disorders and atherogenesis.

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