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Dietary fatty acids influence the appearance of tumour necrosis factor-α receptors on adipocytes following an immune challenge

Hilary A. MacQueen*, Dawn Sadler and Christine Mattacks

Department of Biological Sciences, The Open University, Milton Keynes MK7 6AA, UK

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Rats were fed from weaning on chow supplemented with suet or sunflower oil (10 % (w/w) each). The appearance of receptors for tumour necrosis factor-α on perinodal adipocytes from the popliteal depot following a subcutaneous injection of bacterial lipopolysaccharide was examined. In rats fed on sunflower oil-supplemented chow receptors appeared at a time similar to that described in rats fed unsupplemented chow, but in rats fed on chow supplemented with suet receptor appearance was significantly delayed. The popliteal adipocytes were found to contain different proportions of fatty acids as assessed by GLC. These preliminary results suggest that the fatty acid component of the diet can, by influencing the triacylglycerol-fatty acids within adipocytes, directly alter the time course of an early inflammatory immune response.

Tumour necrosis factor-α receptors: Suet: Sunflower oil: *n*-6 PUFA

In mammals major lymph nodes are always embedded in adipose depots whose mature size changes little with total fatness. This situation contrasts with depots such as the perirenal that are devoid of lymph nodes, and whose mass can change several-fold according to nutritional status (Pond, 1992, 1996*a*,*b*). We have sought a physiological explanation for these anatomical observations, and have suggested that perinodal adipocytes, and to a lesser extent all adipocytes in node-containing depots, are specialised to provision lymph-node lymphoid cells with lipolytic products. These products could serve both as fuel and as precursors for membrane and eicosanoid synthesis (Pond, 1999). *In vivo* and *in vitro* experiments using rats and guinea-pigs have established that lymphoid cells interact strongly with perinodal adipocytes, but do so to a lesser extent with adipocytes from the same adipose depot but located far from the lymph node, and hardly at all with adipocytes from depots that do not contain nodes (Pond & Mattacks 1995, 1998; Mattacks & Pond, 1999). In particular, we have shown that a simulated immune challenge to a popliteal lymph node by subcutaneous injection of bacterial lipopolysaccharide (LPS) stimulates the appearance of receptors for the immune-modulating cytokine tumour necrosis factor (TNF)-α on perinodal adipocytes with a defined time course (MacQueen & Pond, 1998; MacQueen *et al*. 2000).

It is generally accepted that dietary lipids can have important effects on immune function (for example, see Kromann & Green, 1980; Maki & Newberne, 1992), and there is considerable evidence connecting precise dietary components with properties of the immune system (Calder, 1997). It was of interest, therefore, to use our well-characterised experimental system to examine the effect of specific dietary fats on the appearance of TNF-α receptors following an immune challenge. Our model system allows us to examine events at the tissue level *in situ*, and then to isolate the cells and search for molecular correlates of these events, and so represents a powerful approach to investigating the interactions between adipocytes and immune system cells.

Materials and methods

Animals and diets

Rats were CFHB (Wistar-derived) males, between 8 and 9 weeks old at the time of LPS injection. They were bred at the Open University, and kept on a 14 h day–10 h night cycle. They were fed from weaning on RM3 diet (Special Diet Services, Witham, Essex, UK) supplemented with either 10 % (w/w) beef suet (Atora, Hartlepool, Co. Durham, UK) consisting mainly of triacylglycerols containing saturated fatty acids and the monounsaturated fatty acids (MUFA) palmitoleic and oleic acids, or 10 % (w/w) sunflower oil (Tesco, Cheshunt, Herts., UK) consisting mainly of oleic acid and the *n*-6 polyunsaturated fatty acid (PUFA) linoleic acid. The final fatty acid compositions of these diets are shown in Table 1. The RM3 chow was crumbled and mixed with warmed supplement to an even consistency, then allowed to cool and dry for a minimum of 48 h to form a thin cake. This cake was broken into smaller pieces, and fed

Abbreviations: LPS, bacterial lipopolysaccharide; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TNF, tumour necrosis factor.

* **Corresponding author:** Hilary A. MacQueen, fax +44 1908 654167, email h.a.macqueen@open.ac.uk

Table 1. Fatty acid composition (g/100 g total fatty acids) of the experimental diets (Data derived from manufacturers and from Colby & Pond, 1993)

Fatty acid	Unsupplemented chow*	Chow + sunflower oil†	Chow + beef suet‡
Lauric (12:0)	1·3	1·3	1·3
Myristic (14:0)	5·4	4·9	5·3
Myristoleic (14:1)	0·5	0·5	0·5
Palmitic (16:0)	9·7	9·2	11·5
Palmitoleic (16:1)	4·9	4·4	4·7
Stearic (18:0)	2·7	2·6	5·0
Oleic (18:1)	28·3	28·1	29·0
Linoleic (18:2)	34·0	37·1	30·7
Linolenic (18:3)	5·2	4·7	4·7
Arachidonic (20:4)	6·5	5·9	5·9
Docosapentaenoic (22:5)	1·6	1·4	1·4
Total SFA	19·1	18·0	23·1
Total MUFA	33·7	33·0	34·2
Total PUFA	47·3	49·0	42·7

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
* RM3; Special Diet Services, Witham, Essex, UK.
† 10 % (w/w); Tesco, Cheshunt, Herts., UK.
‡ 10 % (w/w); Atora, Hartlepool, Co. Durham, UK.

ad libitum. Diets were made up freshly twice weekly. The rats fed on chow supplemented with suet were heavier at the time of injection (mean mass 365 g; *n* 11) than those fed on chow supplemented with sunflower oil (mean mass 322 g; *n* 12), although they were the same age. This difference was significant ($P < 0{\cdot}01$, *t* test for unmatched samples). The whole popliteal depots of the two groups of animals also differed: suet-supplemented animals had a mean popliteal depot mass of 0·25 g, whereas that from sunflower oil-supplemented animals was 0·21 g. However, there did not appear to be any difference in the size of the lymph nodes themselves in the two groups of animals. There was no difference in the baseline (uninjected) expression level of TNF-α receptors on lymph nodes or adipocytes in these two groups (data not shown). Animals on both diets appeared in good health, although the coats of the suet-fed rats looked and felt greasier than those of animals fed sunflower oil-supplemented or unsupplemented chow.

Treatment with lipopolysaccharide

Animals were weighed immediately before injection. The left popliteal lymph node was activated by injecting LPS subcutaneously (Sigma, Poole, Dorset, UK) at 1 μg/100 g body weight into the left hind lower limb, i.e. distal to the node. This dose is three orders of magnitude below the median lethal dose of 20 mg/kg (Chavali *et al.* 1997), and represents a transient low-level immune challenge (MacQueen & Pond, 1998; MacQueen *et al.* 1999). The treatment produced no apparent discomfort to the animal, and there was no subsequent discolouration or swelling in the injected limb. The right uninjected leg was used as a control. At various times after stimulation of the lymph node, rats were killed by cardiac injection of 1·0–1·5 ml sodium pentobarbitone; 60 mg/ml (Sagatal; Rhône-Mérieux, Harlow, Essex, UK), and the entire popliteal adipose depot, containing its single lymph node, was dissected immediately from each hind limb, weighed, and either wrapped in foil and frozen immediately at –20°C for fatty acid determination or fixed in calcium formal fixative (2 mM-$CaCl_2$ in formalin (10 %, v/v), pH 7·2) for immunohistochemical analysis.

Immunohistochemistry

Samples were treated as described previously (MacQueen & Pond, 1998) to yield sections on slides stained with antibodies to type I TNF-α receptors. The first antibody was goat polyclonal anti-recombinant human TNF soluble receptor type I (R & D Systems, Abingdon, Oxon, UK). The second antibody was fluorescein isothiocyanate-coupled rabbit anti-goat immunoglobulin-γ (Vector Laboratories, Peterborough, UK). Slides were viewed and photographed using an Axiophot fluorescence microscope (Zeiss, Jena, Germany) using a ×10 objective. Film was Kodak T Max P3200. Samples were analysed from eight rats on each diet, two rats at each time point. Results from duplicate animals were similar.

Fatty acid composition analysis

For GLC, whole popliteal depots were removed from animals fed on the two diets, but which had not been injected with LPS, and frozen at –20°C for up to 4 months. On thawing, samples were dissected from the depot. The near-to-node samples were taken from within 2 mm of the lymph node. The far-from-node samples were taken from between 5 and 7 mm from the node. This yielded tissue samples of about 10 mg. Fatty acid analysis was carried out as reported previously (Mattacks & Pond, 1997), except that sample sizes were between 30 and 150 nl. Chromatograms and peak area integrations were produced using JCL 6000 software (Jones Chromatography Ltd, Hengoed, Mid Glamorgan, UK), and manipulation of the data was carried out using Microsoft Excel 98™ (Seattle, WA, USA). Only peaks measuring more than 1 % of the total could be accurately analysed. This system gave a spurious peak of about 1 % at the position expected for 15 : 1; this peak was found regardless of whether or not fatty acid methyl esters or standards

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were loaded, and thus was ignored for the purpose of sample analysis. No peaks were detected above the 18 : 3 peak, suggesting that longer-chain PUFA present in the diet, such as 20 : 4 and 22 : 5, were not incorporated to a level greater than 1 % of the total. Samples for analysis were prepared from two animals on each diet: this low number precluded statistical analysis so data are presented as the mean and range of the two values obtained (see later).

Results

TNF type I receptors are known to first appear 30 min after immune challenge, and increase in number to a peak between 6 and 12 h after immune challenge (MacQueen & Pond, 1998; MacQueen *et al.* 2000). Thus, as expected no receptors were seen on adipocytes at 0 h after LPS injection, and at 0·5 h after injection receptors were seen on adipocytes from sunflower oil-fed, but not from suet-fed rats (not shown). The appearance of immunostained sections taken from rats on the two diets and killed 2 h after LPS injection is shown in Fig. 1. Antibody to TNF receptor type 1 was detected on lymph nodes and perinodal adipose tissue of the rats fed on chow supplemented with sunflower oil. However, in rats fed on chow supplemented with suet there was still no staining on the adipocytes, although the lymph node reacted strongly. However, at 24 h after LPS injection staining was apparent on nodes and perinodal adipocytes from rats on both diets (Fig. 2). Thus, the suet diet appeared to delay the appearance of TNF-α receptors on adipocytes following local LPS challenge.

Analysis of the triacylglycerol-fatty acids contained in adipocytes from rats on both diets revealed that there were substantial differences, both between diets and proximity to lymph nodes. The data are shown in Table 2.

Discussion

Our experiments attempt to shed light on the processes involved when a mammal responds to a minor transient immune challenge, rather than a major disease state. To this end we have devised an *in vivo* system that gives a predictable response to a standard immune challenge, and on which the effects of various dietary components can be assessed. In the present paper we report the effects on this system of two diets, both of which deviate from the recommended amounts of dietary fat for the species, as provided by the unsupplemented diet, yet are not atypical of what wild rats might choose to eat (CM Pond, personal communication). One diet provides an excess of *n*-6 PUFA, the other an excess of saturated fatty acids. In our model system, the diet rich in PUFA supported an inflammatory immune response in a timescale similar to that reported

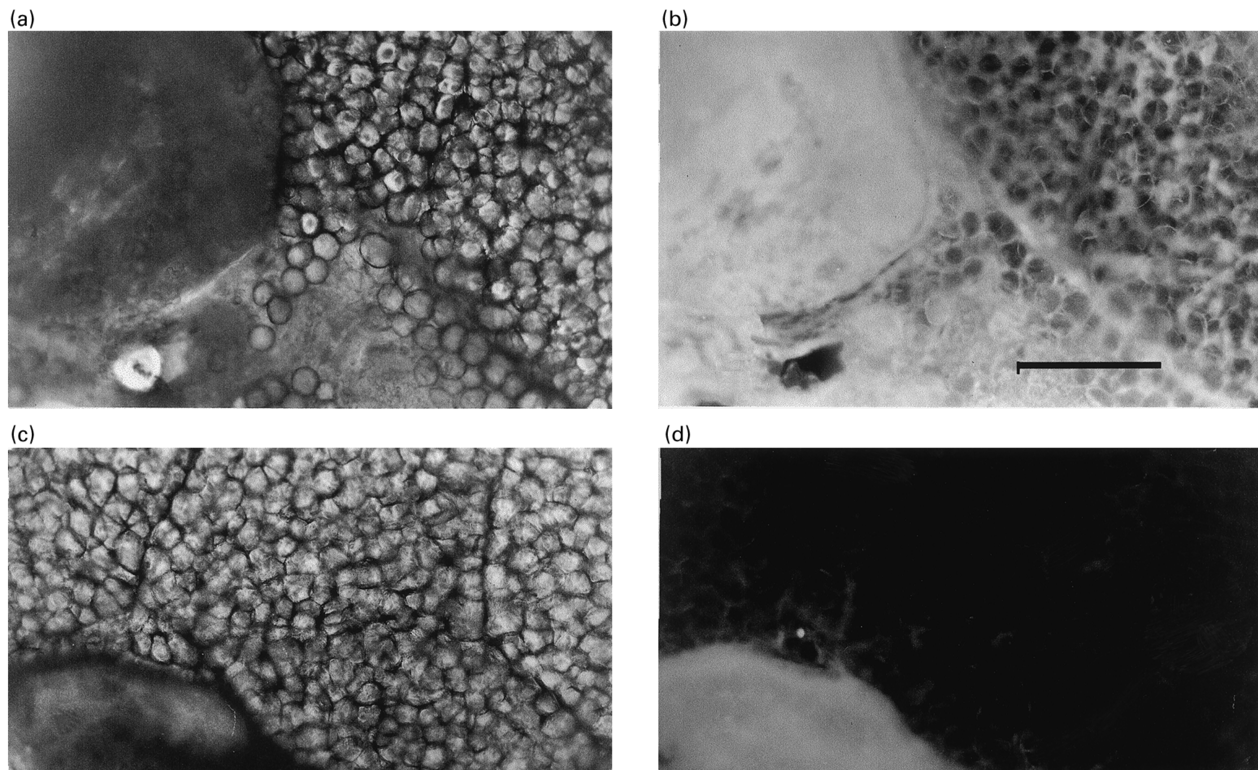

Fig. 1. Appearance of tumour necrosis factor receptors 2 h after an immune challenge (subcutaneous injection of bacterial lipopolysaccharide). (a) Bright field and (b) epifluorescence of lymph node and adipocytes from rats fed on chow supplemented with sunflower oil (10 %, w/w). (c) Bright field and (d) epifluorescence of lymph node and adipocytes from rats fed on chow supplemented with suet (10 %, w/w). Tissue samples were labelled with fluoresceinated anti-tumour necrosis factor receptor type 1 antibody. (a–d) The lymph node is the apparently undifferentiated structure on the lefthand side. The perinodal adipocytes are large round cells and can be seen clearly to be in intimate contact with the node. For details of diets and procedures, see pp. 387–389. (scale bar 0·5 mm)

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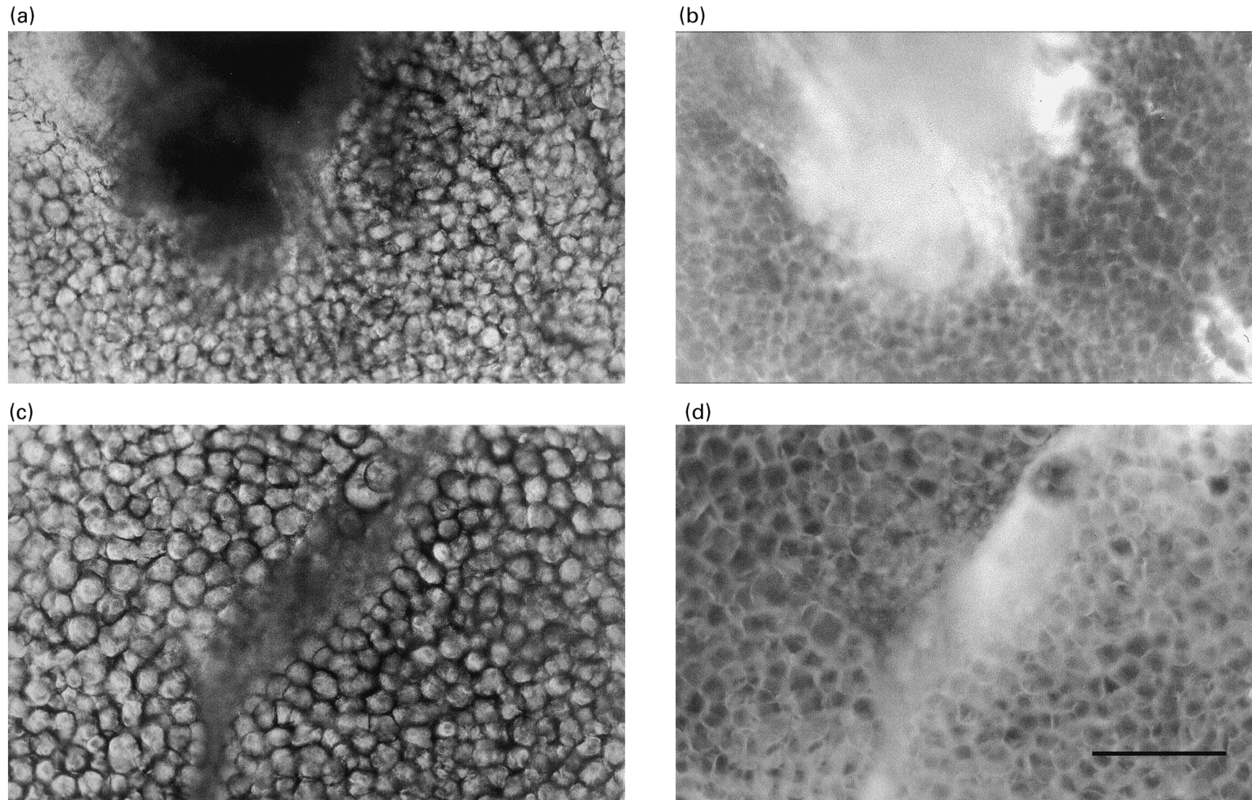

Fig. 2. Appearance of tumour necrosis factor receptors 24 h after an immune challenge (subcutaneous injection of bacterial lipopolysaccharide). (a) Bright field and (b) epifluorescence of lymph node and adipocytes from rats fed on chow supplemented with sunflower oil (10 %, w/w). (c) Bright field and (d) epifluorescence of lymph node and adipocytes from rats fed on chow supplemented with suet (10 %, w/w). Tissue samples were labelled with fluoresceinated anti-tumour necrosis factor receptor type 1 antibody. (a, b) The lymph node is the structure seen at the centre top. (c, d) The tip of the lymph node can be seen running from top to bottom across the centre. The large, rounded adipocytes can clearly be seen to be in intimate contact with the node. For details of diets and procedures, see pp. 387–389. (scale bar 0·5 mm)

for rats on an unsupplemented diet (MacQueen & Pond, 1998; MacQueen *et al.* 2000). However, the diet enriched in saturated fatty acids delayed this aspect of adipocyte involvement in the immune response. A similar level of receptors was achieved with both diets 24 h after the immune challenge. As yet we have no data relating to the intervening time points, but an ongoing follow-up study should allow us to establish a time course for receptor

Table 2. Fatty acid composition (g/100 g total fatty acids) of samples of popliteal adipose tissue taken from rats fed on diets supplemented with suet (10 %, w/w; suet-fed) or sunflower oil (10 %, w/w; sunflower-fed)*

(Values are means and ranges for two animals)

	Suet-fed				Sunflower-fed			
	Near-to-node		Far-from-node		Near-to-node		Far-from-node	
Fatty acids	Mean	Range	Mean	Range	Mean	Range	Mean	Range
14:0	4·3	4·1–4·6	4·3	4·3–4·3	4·1	4·9–3·3	2·5	2·0–3·0
15:0	nd		4·0	4·0–4·0	nd		nd	
16:0	34·5	37·1–31·9	37·1	37·1–37·0	31·1	31·5–30·6	34·6	33·3–36·0
16:1	5·3	5·2–5·4	7·0	7·0–7·0	4·7	5·1–4·4	4·6	4·5–4·6
18:0	2·0	1·9–2·1	3·5	3·5–3·5	1·5	1·5–1·4	2·0	1·9–2·2
18:1	28·5	27·4–29·6	39·1	39·1–39·1	23·1	23·3–23·0	27·6	25·9–29·4
18:2	19·9	18·9–20·9	3·2	3·1–3·2	26·6	24·9–28·3	22·9	24·7–21·0
18:3	4·3	4·2–4·4	nd		8·4	8·9–7·9	5·8	7·7–3·9
Total saturates	40·8		48·9		36·7		39·1	
Total unsaturates	58·0		49·3		62·8		60·9	
Total MUFA	33·8		46·1		27·8		32·2	
Total PUFA	24·2		3·2		35·0		28·7	
MUFA : PUFA	1·4		14·4		0·8		1·1	

nd, Not detected; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
* For details of diets and procedures, see pp. 387–389.

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appearance on the two diets. We do not yet know whether the lag in receptor appearance is due to a delay in receptor gene expression, protein synthesis or receptor assembly, and we are currently investigating these factors. We are also considering the possible involvement of peroxisome proliferator-activated receptor-γ, a transcription factor known to mediate many adipocyte functions (for review, see Lowell, 1999).

When we examined the composition of the triacylglycerol-fatty acids within the perinodal adipocytes we found that in general saturated fatty acids are distributed fairly evenly among the four samples. An exception is 15 : 0, which is a product of the rumen microflora (Christie, 1981), and is present only in the animals fed beef suet. All the 15 : 0 is sequestered to the far-from-node adipocytes. Considering the MUFA, in suet-fed animals rather more MUFA are found in far-from-node samples (46·1 g/100 g total fatty acids compared with 33·8 g/100 g total fatty acids in near-to-node samples), whereas in sunflower oil-fed animals the distribution is generally more even (32·2 and 27·8 g/100 g total fatty acids respectively). A different pattern is seen with the PUFA 18 : 2 and 18 : 3. In rats fed the sunflower oil-supplemented diet, with plenty of available PUFA, the distribution of these fatty acids is broadly similar in near-to-node (35 g/100 g total fatty acids) and far-from-node (28·7 g/100 g total fatty acids) samples. However, in animals fed on suet-supplemented diets, where PUFA are relatively rare, there was a marked difference, with 24·2 g/100 g total fatty acids in the near-to-node samples compared with 3·2 g/100 g total fatty acids, and no detectable 18 : 3 in the far-from-node samples, suggesting that in these animals approximately all the available PUFA were found in near-to-node samples. This result may suggest that in animals fed suet-supplemented chow there was a deficiency of 18 : 3. MUFA : PUFA is ten-fold higher far from the node compared with near the node in the suet-fed rats, but less than two times greater in the sunflower oil-fed animals. These results largely confirm the pattern found in guinea-pigs (Mattacks & Pond, 1997), except that in these animals there was no change in MUFA distribution.

The site specificity of triacylglycerol-fatty acids distribution found in animals on both diets suggests an active mechanism for sequestration of particular fatty acids, although the mode of action of any such mechanism is presently unknown. Selective incorporation of particular fatty acids has long been known (Lands *et al.* 1982), and Raclot and colleagues (Raclot & Groscolas, 1993; Raclot *et al.* 1995*a*,*b*) have suggested that there may be a mechanism for differential fatty acid mobilisation based on differences in chain length and number of double bonds. Differences in enzyme specificity may also play a role in generating the variety of gradients shown by our data.

The sunflower oil used in these experiments contained large amounts of *n*-6 PUFA. Dietary *n*-6 PUFA have been reported to be ‘pro-inflammatory’ (Grimble & Tappia, 1995; Calder, 1998), and indeed the sunflower-supplemented diet used here appears to support an immune response (as judged by the appearance of TNF receptors on responding adipocytes) in the time frame expected from animals fed on a control diet. In contrast, epidemiological data suggest that the consumption of *n*-3 PUFA-rich diets is associated with an absence of inflammatory disorders, and Calder (1998) has reviewed evidence suggesting that *n*-3 PUFA suppress the inflammatory immune response, although this view has been criticised (Netea *et al.* 1999). It would be interesting to discover whether a diet rich in *n*-3 PUFA (such as would be found in fish oils) would alter the time course of the inflammatory response in our model system.

The overall fatty acid distribution in near-to-node samples does not change a great deal with diet. What does change markedly, however, is the receptor response time. Dietary fatty acids could affect the receptor response time via changes in membrane fluidity (Parrish *et al.* 1997; Tappia *et al.* 1997), or by changes in lipid domain structure (Clamp *et al.* 1997). This possibility assumes that phospholipid-fatty acids are altered in a similar way to triacylglycerol-fatty acids in our system, as has been reported elsewhere (Khuu Thi-Dinh *et al.* 1990; Clamp *et al.* 1997; Parrish *et al.* 1997). TNF is a trimer, and its binding to one receptor molecule leads to subsequent clustering of further receptor molecules. This clustering leads to receptor activation (Van den Abeele *et al.* 1995). The movement of receptor molecules within the phospholipid bilayer may be affected by specific fatty acyl components of the bilayer. Alternatively, the effect might be at the level of receptor protein synthesis, or even gene expression. There have been several reports (for review, see Calder, 1997) that dietary fatty acids can affect the production of leukotrienes and other eicosanoids, which classes of compounds are known to affect gene expression (Smith & Borgeat, 1985). At a different level, it is possible that TNF receptor appearance on adipocytes is directly proportional to the amount of TNF-α produced by the lymphoid cells to which they are responding, and animals fed an excess of saturated fatty acids are unable to produce TNF-α in the normal time frame. We are currently investigating these possibilities.

Finally, we can speculate on the precise role of TNF receptors on adipocytes. They are known to play a role in mediating insulin resistance, including the transient insulin resistance that occurs during an acute immune response (for review, see Peraldi & Spiegelman, 1998; Del Aguila *et al.* 1999). They may also serve other functions, such as allowing the adipocytes to respond to an immune challenge by provisioning the immune system (Pond, 1996*a*, *b*), and ‘mopping up’ excess TNF-α to prevent an overreaction of the inflammatory immune response (van Zee *et al.* 1992). In this context it has recently been reported that in mice injected with LPS plasma concentrations of TNF-α peak at 90 min then drop quickly (Sadeghi *et al.* 1999). This finding may reflect binding to TNF receptors produced in a time frame similar to that which we have observed. In conclusion, the experiments reported here are consistent with the hypothesis that perinodal adipocytes play a key role in the natural regulation of lymph-node immune responses.

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