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Immunofluorescent localisation of tumour necrosis factor-α receptors on the popliteal lymph node and the surrounding adipose tissue following a simulated immune challenge

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ABSTRACT

We used immunohistochemical techniques to demonstrate the distribution of receptors for the cytokine tumour necrosis factor-α on the popliteal lymph node and the adipose tissue surrounding it for 5 d following a simulated immune challenge to one hind leg in the rat. We found different patterns of expression of receptors on adipocytes surrounding a lymph node to a distance of about 1 mm, and on those more remote from the node. Sites recognised by an antibody to type I tumour necrosis factor receptors appeared on the challenged node and the adipocytes surrounding it within 30 min of an injection of bacterial lipopolysaccharide, but appeared on adipocytes surrounding the unchallenged popliteal node only 24 h later. Adipocytes distant from the node, both within the same depot and in the contralateral depot, showed no response. Sites recognised by an antibody to type II tumour necrosis factor receptors were present at all times on lymph nodes and the adipocytes close to them, but appeared on more distant adipocytes only 24 h after immune challenge, in both challenged and unchallenged legs. These data support the proposal, based on in vitro studies, that the adipose tissue surrounding major lymph nodes is specialised to respond to cytokines derived from lymphoid cells, and participates in the immune responses of the adjacent node.

Key words: Adipocytes; lymphoid cells; immune response; cytokines.

INTRODUCTION

In mammals, localised infections elicit a rapid immune response in lymph nodes that acts as a first line of defence. These nodes are always surrounded by adipose tissue. Even in animals undergoing natural fasts or maintained on severely restricted diets, there remains a layer of adipose tissue around lymph nodes that appears to be protected from the general mobilisation of fat reserves observed during starvation or fever (Pond, 1996*a*, *b*). We have sought an explanation for this close anatomical association between adipose tissue and lymph nodes, and in vitro experiments (Pond & Mattacks, 1995; Mattacks & Pond, 1997) have demonstrated that the adipose tissue around lymph nodes has properties which would enable it to participate in local interactions with lymph node lymphoid cells. When explants of adipose tissue taken from a depot surrounding a lymph node are cocultured with lymph node lymphoid cells, lipolysis from near-to-node adipocytes increases more than 3-fold, while that from adipose tissue from elsewhere in the same depot barely doubles. Triacylglycerols from adipose tissue near to lymph nodes contains a greater proportion of polyunsaturated fatty acids (PUFAs) than those remote from the node (Mattacks & Pond, 1997). As well as serving as fuel, these FAs act as precursors for plasma membrane biosynthesis, and certain FAs are also precursors of eicosanoids and prostaglandins. It seems possible therefore that the local availability of a particular mixture of FAs, released by adipocytes in response to some signal from the lymph node, may stimulate or inhibit the production of further signal molecules by the lymph node lymphoid cells, as well as supplying necessary materials for cell proliferation, resulting in a prompt, substantial immune response that may nonetheless be kept at a local level, or extended systemically.

Interactions between lymphoid cells, and between

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the immune system and other types of cell, are frequently mediated by cytokines. One of the most important mediators in the early immune response is tumour necrosis factor alpha (TNFα), but an excess in the circulation can have serious physiological consequences such as toxic shock syndrome (Tracey & Cerami, 1992). Interactions involving immune cells may involve systemic, blood-borne stimuli, but they can also take place at a local level. TNFα, produced by many cell types including adipocytes and several populations of immune cells, has been implicated in the development of fever and subsequent cachexia (Waage, 1992). In view of the site-specific capacity of adipose tissue to respond to local signals from lymph node cells (and possibly from themselves), it was of interest to examine the anatomical position and time-course of appearance of TNF receptors in the adipose tissue around lymph nodes after an immune stimulus.

There are 2 types of receptor to TNFα, and they appear to mediate different intracellular response pathways. TNF receptor I (p55, CD120a) primarily mediates the growth inhibitory (Carter et al. 1996) and apoptotic (Tartaglia et al. 1993) responses, whereas several reports of actions involving the immune system by TNFα implicate the type II (p75, CD120b) receptor (e.g. Abe et al. 1995). Many clinical studies, for example those of Steinshamn et al. (1996) and Herbein et al. (1996) show differential effects of stimulation via the 2 receptor types, but as yet little is known about the mechanisms involved (reviewed by Aggarwal & Natarajan, 1996).

We wished to discover whether the distribution of membrane receptors for immunoactive substances such as TNFα might be correlated with the site-specific differences in the capacity of adipose tissue to interact with lymphoid cells in vitro. We chose the popliteal adipose depot for this investigation because it is easily dissected, and the single lymph node within it is relatively large and constant in position. The node drains the whole lower hind limb, and so a local immune response similar to that to a natural infection can be elicited in it by injecting a low dose of bacterial lipopolysaccharide (LPS), a known elicitor of TNFα (Old, 1985), into the lower limb. If only one leg is stimulated, then the contralateral depot in the same animal can be used as an unstimulated control, thereby eliminating much between-animal variation.

MATERIALS AND METHODS

Rats were CFHB (Wistar-derived) males, aged 8–9 wk, and of body mass 350–450 g at the time of injection. They were bred at the Open University, kept on a 14 h day–10 h night cycle, and fed RM3 diet. The rats appeared to be in good health, but were not raised in specific pathogen-free conditions.

In rats of the size we used, the popliteal depot is approximately pyramidal in shape, about 10 mm high with a base whose sides are $\sim$ 5 mm long. The volume of the depot is therefore less than 100 mm^3, of which the lymph node, sited near to the posterior ventral corner, occupies about 17%, being ovoid in shape, approximating to a sphere of diameter 3 mm. The distance between the lymph node and the nearest edge of the depot is at least 1 mm, and in some directions approaches 3–4 mm. The diameter of adipocytes at these sites is between 0.07 and 0.1 mm, so there is a minimum of 10 cells between the node and the nearest edge, and there may be more than 50. Adipocytes were defined as 'near' if they were within 1 mm of a node, and 'far' if they were more than 3 mm from a node.

The left popliteal lymph node was activated by injecting lipopolysaccharide (LPS) (Sigma UK, Poole) subcutaneously at approximately 1 μg per 100 g body weight into the lower left hind limb, i.e. distal to the node. This treatment produced no apparent discomfort to the animal, and there was a complete absence of subsequent discolouration, swelling or soreness in the injected limb.

The time points used were 0, 0.5, 1, 2, 6, 12, 18, 24, 48, and 120 h after the simulated immune challenge. Rats were killed at the above times by cardiac injection of 1.0–1.5 ml of 60 mg/ml sodium pentobarbitone (Sagatal, Rhône-Mérieux, Ireland), and the entire popliteal adipose depot, each containing its single lymph node, was dissected immediately from each hind limb, weighed, and fixed in calcium formol fixative (2 mM calcium chloride in 10% formalin, pH 7.2). They were kept at +4 °C until sectioned.

Sectioning was done using a Vibratome Series 1000 (General Scientific, Redhill, UK), with a nominal section thickness of 120 μm. This thickness, being 20% more than the diameter of the largest adipocytes, permitted good visualisation of the material, without rupturing too many adipocytes. Sectioning was carried out on a bed of dry ice to solidify the tissue. Thawed slices on microscope slides showed no evidence of internal damage or distortion. The slides were precoated with poly-L-lysine.

The antibodies we used were polyclonal anti-recombinant human TNF soluble receptor types I and II (anti-TNFRI and anti-TNFRII), raised in goat and purchased from R & D Systems, Abingdon, UK, catalogue nos AB-225-PB and AB-226-PB respectively. These antibodies cross-react with rodent TNF

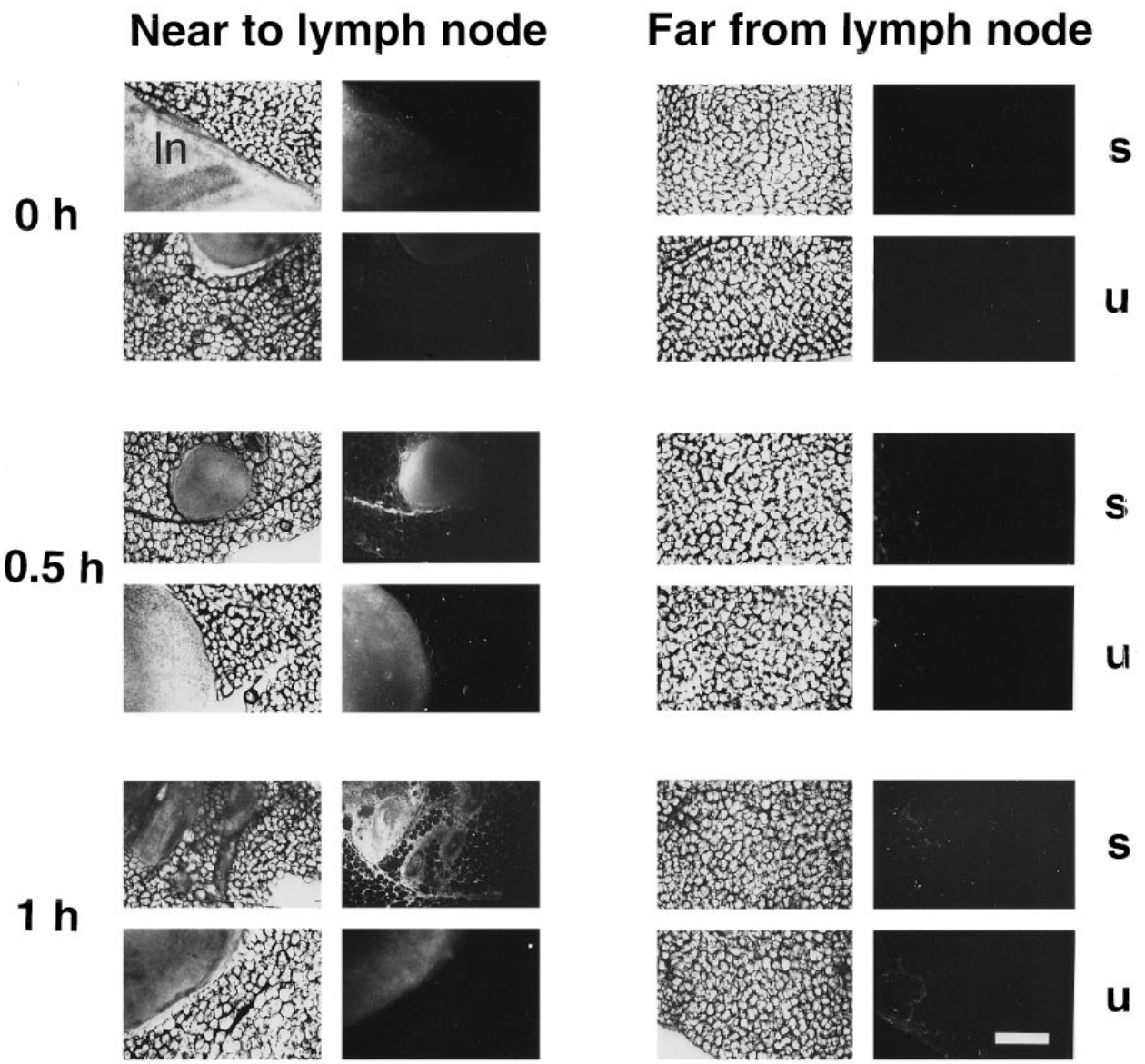

Fig. 1. Immunofluorescence shown by lymph nodes and adipocytes treated with antibody to TNFRI at 0, 0.5 and 1 h after LPS injection. Left hand columns: lymph node and immediately surrounding adipose tissue; right hand columns: adipose tissue distant from lymph nodes. In each pair of photographs the left is the bright field, the right is the fluorescence shown by the same field. The line of fluorescence seen in the 0.5 h stimulated sample is due to TNF receptors on the endothelium of a small vessel, seen also in the bright field picture. s, stimulated depot; u, unstimulated depot; ln, lymph node. Bar, 0.5 mm.

receptors (R & D Systems product information). Lyophilised antibodies were suspended in 25 mM phosphate-buffered saline (PBS) at pH 7.2, aliquoted and stored at −20 °C, as recommended by the manufacturers. The second antibody was FITC-coupled rabbit antigoat IgG, from Vector Laboratories, Peterborough, UK; catalogue number FI-5000. Incubations were carried out overnight at +4 °C, after each incubation. Control incubations using biopsy samples of human tissue showed a similar level of specific fluorescence to that shown by rat tissues (data not shown). In all cases, immunofluorescence was completely abolished by preincubation of the first antibodies with the appropriate purified receptor protein (data not shown), suggesting that the fluorescence we saw represented a specific immunoreaction to TNFα receptors. We did not routinely use a blocking solution as in our system the second antibody always showed negligible nonspecific binding.

Slides were viewed and photographed using an Axiophot fluorescence microscope (Zeiss) using a ×10 objective. Film was Kodak T Max P3200. Fields were selected to avoid the many blood and lymphatic

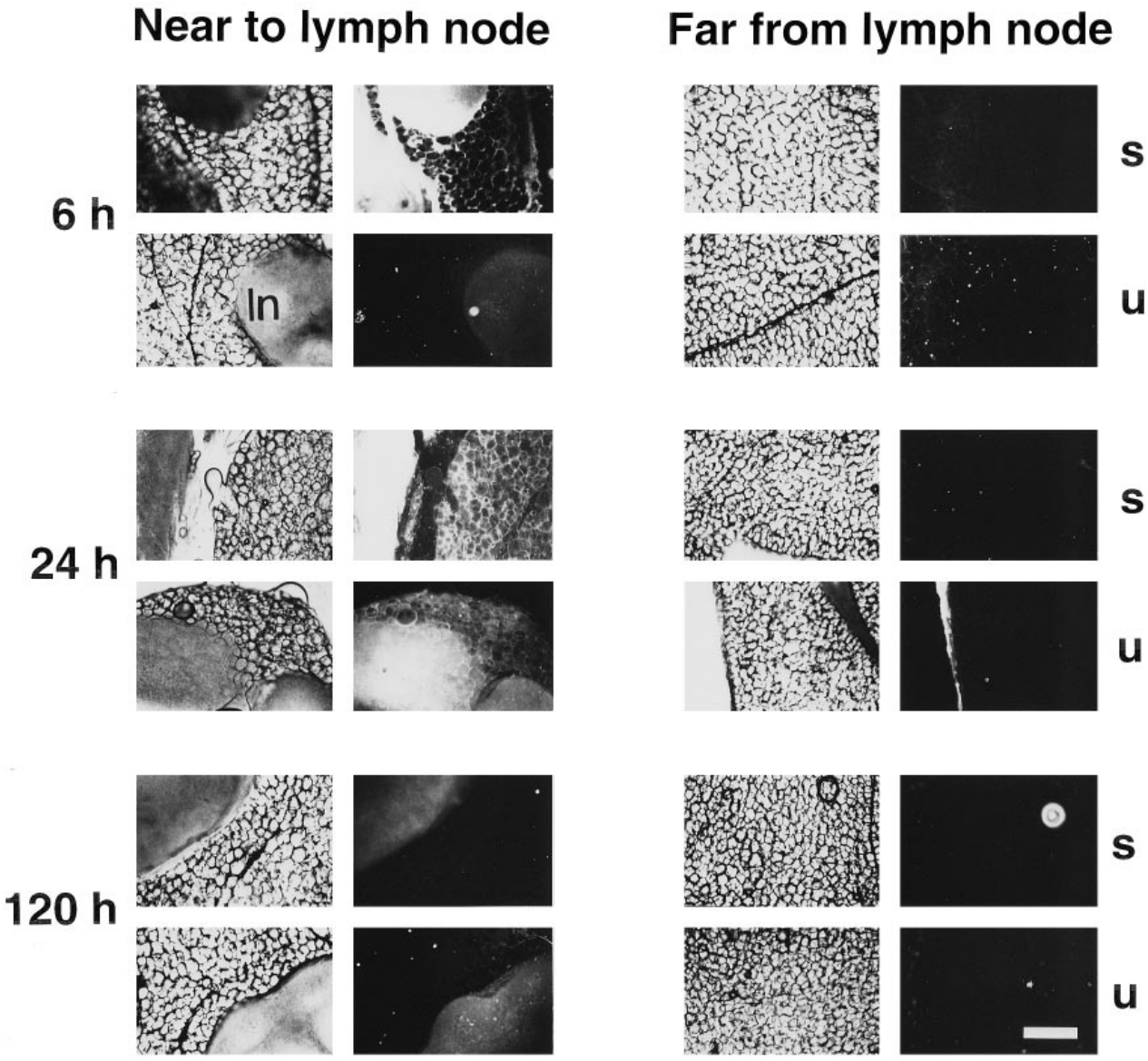

Fig. 2. Immunofluorescence shown by lymph nodes and adipocytes treated with antibody to TNFRI at 6, 24 and 120 h after LPS injection. Left hand columns: lymph node and immediately surrounding adipose tissue; right hand columns: adipose tissue distant from lymph nodes. In each pair of photographs the left is the bright field, the right is the fluorescence shown by the same field. s, stimulated depot; u, unstimulated depot; ln, lymph node. Bar, 0.5 mm.

vessels, which fluoresced brightly owing to the high concentrations of TNF receptors on their endothelia (Slowik et al. 1993). This bright fluorescence interfered with the photographing of fluorescence on the adipocytes. In selecting fields, adipocytes near to the node were photographed along the sides of the node most distant from an edge, and those far from the node were selected along this same vector, so that there was a clear anatomical separation between near and far adipocytes. All animals in a treatment group (between 2 and 6 for different groups) showed similar results, although a subjective estimation of the intensity of fluorescence suggested that the extent of response was not always identical.

RESULTS

The presence of blood vessels throughout the adipose depot provided a useful positive control for each slide: blood vessel endothelia are known to be rich in TNFα receptors of both types (Slowik et al. 1993), and indeed even microvessels always showed strong fluorescence (see e.g. Fig. 1, 0.5 h stimulated sample).

Anti-TNFRI

Figures 1 and 2 demonstrate that immunofluorescence was not apparent in the lymph nodes at 0 h, but faint immunofluorescence was apparent from 0.5 h on both

Table 1. *Summary of immunofluorescence seen on lymph nodes and adipose tissue after treatment with an antibody to TNFRI*

Time after injection (h)	n*	Lymph node		Adipocytes near node		Adipocytes far from node	
		Stim	Unstim	Stim	Unstim	Stim	Unstim
0	2	−	−	−	−	−	−
0.5	4	+	+	+	−	−	−
1	3	+	+	+	−	−	−
2	6	+	+	+	−	−	−
6	4	+	+	+	−	−	−
12	2	+	+	+	−	−	−
18	4	+	+	+	−	−	−
24	2	+	+	+	+	−	−
48	6	+	+	+	+	−	−
120	4	+	+	−	−	−	−

* Number of animals.

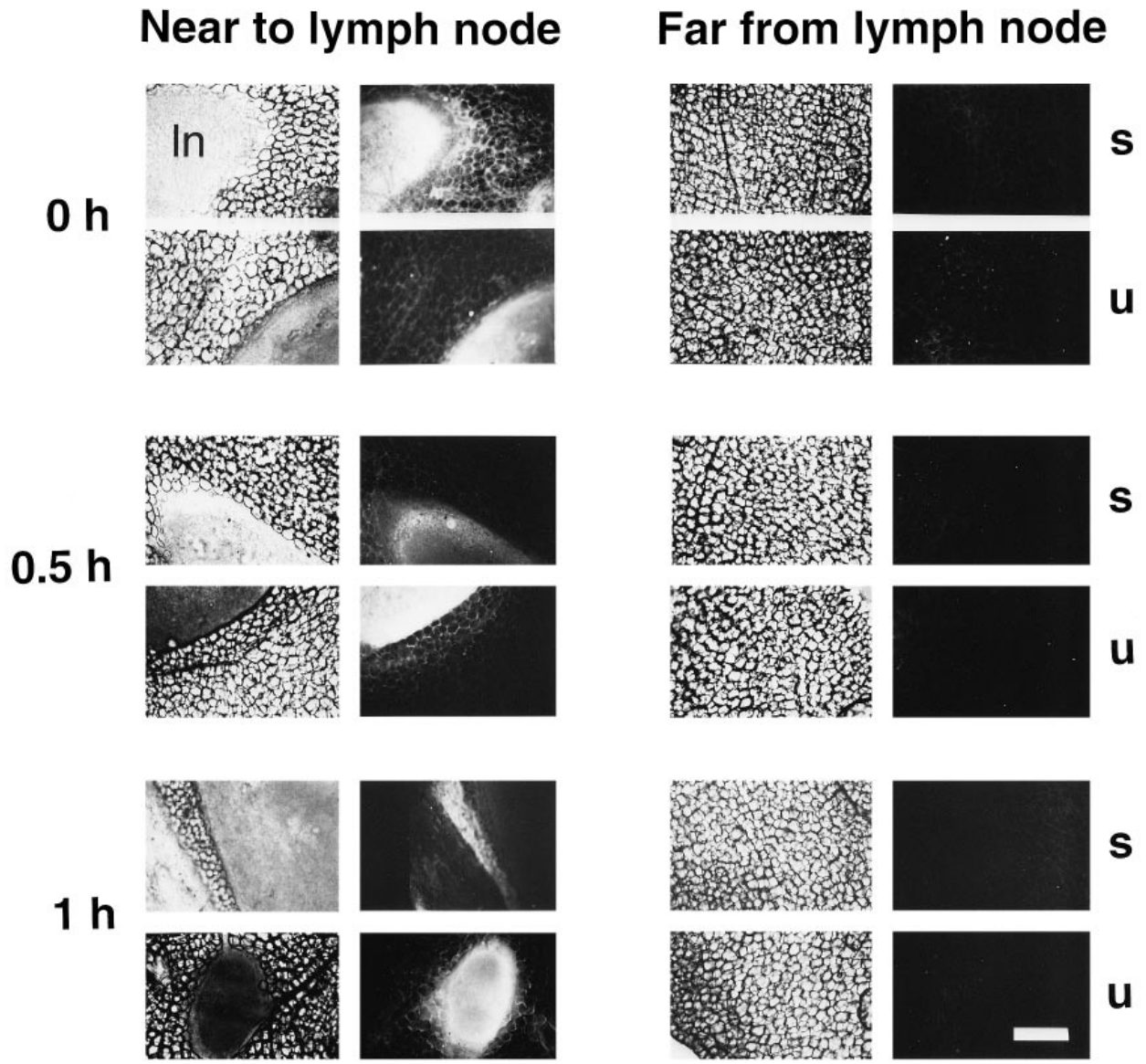

Fig. 3. Immunofluorescence shown by lymph nodes and adipocytes treated with antibody to TNFRII at 0, 0.5 and 1 h after LPS injection. Left hand columns: lymph node and immediately surrounding adipose tissue; right hand columns: adipose tissue distant from lymph nodes. In each pair of photographs the left is the bright field, the right is the fluorescence shown by the same field. s, stimulated depot; u, unstimulated depot; ln, lymph node. Bar, 0.5 mm.

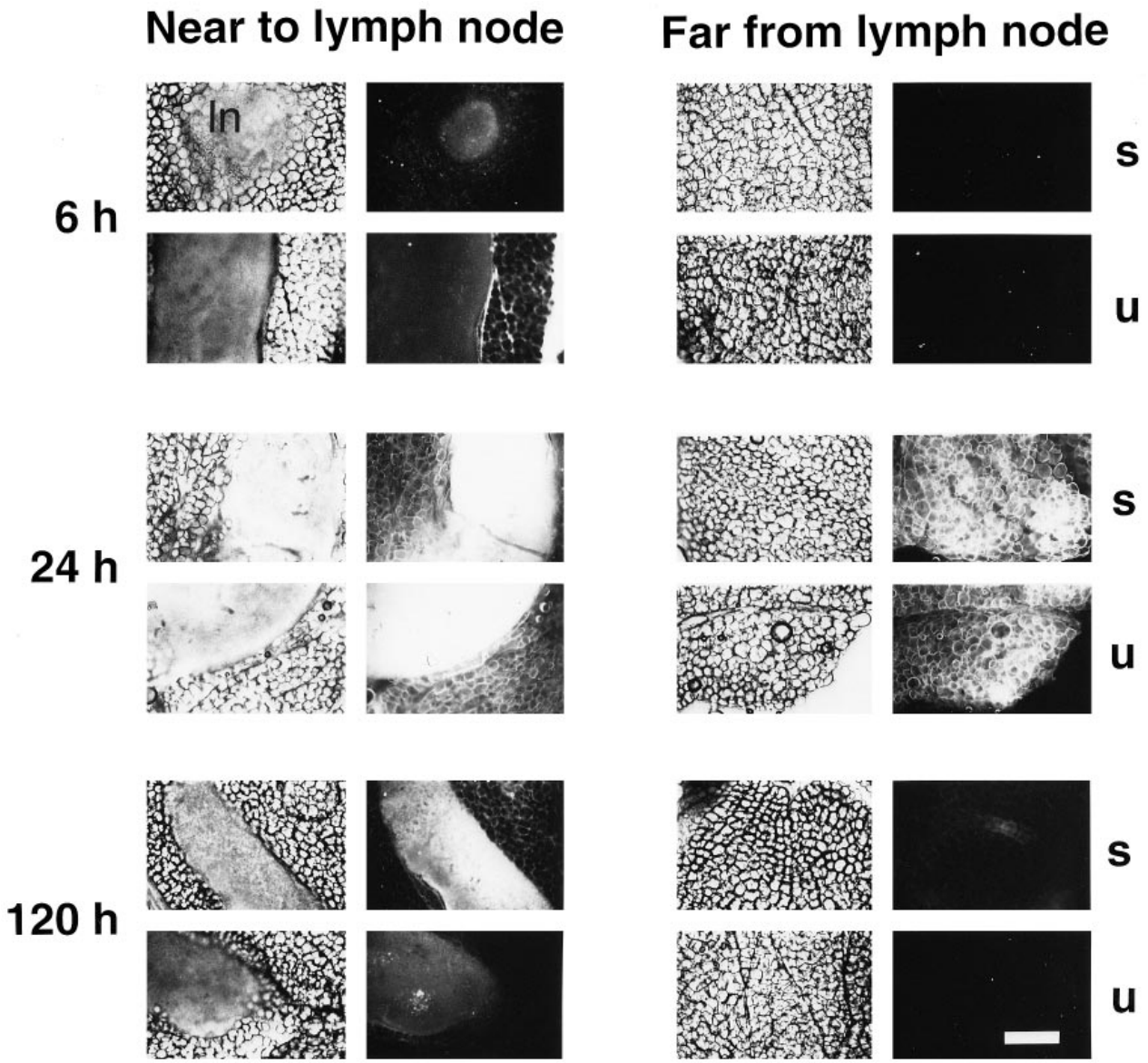

Fig. 4. Immunofluorescence shown by lymph nodes and adipocytes treated with antibody to TNFRII at 6, 24 and 120 h after LPS injection. Left hand columns: lymph node and immediately surrounding adipose tissue; right hand columns: adipose tissue distant from lymph nodes. In each pair of photographs the left is the bright field, the right is the fluorescence shown by the same field. s, stimulated depot; u, unstimulated depot; ln, lymph node. Bar, 0.5 mm.

stimulated and unstimulated nodes, and persisted throughout the period of examination (120 h). In the stimulated leg, there was detectable immunofluorescence at 0.5 h after injection on the adipocytes near to, but not on those far from, the node. This fluorescence was maximal after 1 h, and persisted for several days on adipocytes near the node, but had disappeared by 120 h after injection. In the unstimulated leg no fluorescence was seen on adipocytes near the node until 24 h after injection, when there was a transient appearance of fluorescence that persisted to 48 h, but had disappeared by 120 h. Adipocytes distant from the node showed no fluorescnece either in the stimulated or the unstimulated leg. In the interests of clarity, patterns of fluorescence are shown in Figures 1 and 2 for only 6 time points (0, 0.5, 1, 6, 24 and 120 h after injection), but all data are summarised in Table 1.

Anti-TNFRII

Figures 3 and 4 show that with this antibody fluorescence is found both on the stimulated and unstimulated lymph nodes at all time points. Fluorescence was also apparent on adipocytes near to the node in both legs at 0 h, and persisted over the time period studied. Although it is impossible to quantify fluorescence by these techniques, there appeared to be

Table 2. *Summary of immunofluorescence seen on lymph nodes and adipose tissue after treatment with an antibody to TNFRII*

Time after injection (h)	n*	Lymph node		Adipocytes near node		Adipocytes far from node	
		Stim	Unstim	Stim	Unstim	Stim	Unstim
0	2	+	+	+	+	−	−
0.5	4	+	+	+	+	−	−
1	3	+	+	+	+	−	−
2	6	+	+	+	+	−	−
6	4	+	+	+	+	−	−
12	2	+	+	+	+	−	−
18	4	+	+	+	+	−	−
24	2	+	+	+	+	+	+
48	6	+	+	+	+	+	+
120	4	+	+	+	+	−	−

* Number of animals.

consistently brighter labelling at 24 h after injection than at any other time. In contrast, on adipocytes far from the node, immunofluorescence was absent at early stages, but then appeared transiently and brightly at 24 h after injection in both legs. This fluorescence was still there at 48 h (not shown), but had disappeared by 120 h. Fluorescence on the lymph node cells had a punctate appearance that was not found with the antibody to TNFRI. Again, only the key time points are shown in Figures 3 and 4; the whole set of data is shown in Table 2.

DISCUSSION

Sites reacting with antitype II (p75, CD120b) receptor antibodies are always present in lymph nodes and on the adipocytes near to them (Table 2) and are therefore likely candidates to mediate early steps in an immune response, as discussed by Abe et al. (1995). On the other hand, sites reacting with antitype I (p55, CD120a) receptor antibodies are absent in the quiescent state, but appear on lymph nodes and near-to-node adipocytes within 30 min of an immune challenge (Table 1). Both types of presumed receptor appear on adipocytes following LPS exposure in a pattern that depends on immune status, time, and proximity of the adipocytes to a lymph node. Two patterns are seen. The first seems to represent an early response to immune challenge, occurring as it does within 30 min of that challenge for type I receptors (Figs 1, 2; Table 1). This response persists for at least 2 d but less than 5 d and may be mediated by local diffusion of a signal from the node or node vessels (see below), or even by TNFα produced by the adipocytes themselves. The second pattern, shown by type II receptors, occurs between 18 and 24 h after injection, affects previously unaffected adipocytes, and is probably mediated by systemic processes (Figs 3, 4; Table 2). It seems likely that some signal must enter the general circulation in a sufficient concentration to activate the adipose tissue surrounding the contralateral popliteal lymph node, and presumably also the adipose tissue surrounding lymph nodes elsewhere in the body. This interpretation supports previous observations that adipose tissue around all major lymph nodes has much greater capacity to respond to signals arising from immune cells (Pond & Mattacks, 1995). Figure 4 suggests that whatever signal the adipocytes are responding to has reached all parts of the contralateral depot by 24 h after injection, and that all the popliteal adipocytes are competent to respond by producing type II receptors, though not type I. Interestingly, in vitro experiments show that popliteal adipose tissue remote from the node responds quite strongly to the presence of lymphoid cells, compared with far-from-node samples from other node-containing depots such as inguinal, and much better than samples from nodeless depots such as perirenal (Pond & Mattacks, 1995).

Diffusion of cytokines from lymph nodes or the vessels supplying them has not been measured directly. Adipose depots and the nodes within them are well supplied and drained by blood and lymph vessels (reviewed by Heath et al. 1995 and Hay & Young, 1995). The afferent and efferent vessels pass through the adipose tissue to and from the lymph node. Vessels branch close to the surface of the lymph node, and branching continues within the node so that it is extensively vascularised throughout. However, there is considerable microvascular branching within the adipose tissue as well (MacQueen & Pond, unpublished results), and it seems likely that material diffusing from the vessels could easily reach all parts of the depot. It therefore appears that the adipocytes

are responding differently not because of some artefact of diffusion but because of the site-specific properties of the adipocytes themselves.

It is not clear what significance can be attached to the different patterns shown by the 2 types of antireceptor antibody, but our results are consistent with the view that they mediate different cellular processes. The appearance of presumed type I receptors, involved in growth retardation and apoptosis (Carter et al. 1996; Tartaglia et al. 1993), within 30 min of immune challenge, suggests that induction of processes occurring by related intracellular mechanisms in nearby adipocytes may be an early part of the immune response. Presumed type II receptors are present constitutively on lymph nodes and near-to-node adipocytes, but appear on far-from-node adipocytes only 24 h after an immune challenge, suggesting that they mediate different responses. TNF has a variety of effects on adipose tissue, the most notable being lipolysis (Grunfeld et al. 1996; Declercq et al, 1996; Zhang et al. 1996), although only one study (Hofmann et al. 1994) has related these effects to receptor expression. In normal developmental processes, such as development of the mammary gland, the 2 types of receptor are known to play different but complementary roles (Varela & Ip, 1996), and TNFα receptors are differentially expressed on adipose tissue in obese humans and rats (Hotamisligil et al. 1997), so it would not be surprising if that had different functions in the early immune response.

It has long been believed that the lipid component of the diet influences many aspects of immune system activity, but the mechanisms involved are unclear. Differences in phospholipid FAs may affect membrane fluidity and receptor mobility (Tappia et al. 1997) and it is possible that changes in these factors could affect the aspects of the immune response that are receptor-mediated. Many dietary and in vitro experiments have demonstrated effects of FAs, particularly PUFAs, on immune cell function (e.g. Yaqoob & Calder, 1995; Ferrante et al. 1997; Tappia et al. 1997). FAs from triacylglycerols are precursors for the synthesis of membrane phospholipids, and PUFAs derived from membrane phospholipid FAs are themselves precursors for the second messenger arachidonic acid and for eicosanoids, so this is another route by which dietary lipids might affect immune function. The greater abundance of PUFAs in adipocytes near lymph nodes reported by Mattacks & Pond (1997) suggests that lipolysis induced in them by TNFα could give lymphoid cells priority access to PUFAs that they need for synthesis and for immunomodulatory activities. We suggest that one aspect of the early immune response by lymph node lymphoid cells relates to their close association with the adipose tissue in which they are embedded. The relationship is complex, but may involve cytokines such as TNFα, known to be an early response to LPS-induced immune challenge, passing from lymph node cells to adipocytes, possibly via the thin-walled, highly branched blood and lymphatic vessels. The adipocytes then may respond by releasing other molecules which interact with the lymph node cells at a local level. Some of these molecules may be PUFAs from triacylglycerols that can act not only as precursors for the new membranes synthesised by proliferating lymphoid cells, but also as precursors for intracellular messenger molecules which modulate the immune response.

We conclude that the adipose tissue around the popliteal lymph node is actively recruited to an immune response in that node. The adipocytes in a small region of the depot are specifically equipped to respond rapidly, by the pathways induced by activation of type I receptors, to the TNFα released after immune stimulation, whereas those elsewhere in the depot lack this ability. Our results suggest a physiological explanation for the long-known, ubiquitous and intimate anatomical relationship between lymph nodes and adipose tissue.

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