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Quantitative Analysis of Cytokine Receptors Using Single-Photon Counting

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Fluorescent molecules are widely used as labels or probes in biological research [1,2]. In particular, they can be chemically attached to antibodies which, by binding to their specific antigens, can identify the location of particular molecules of biological interest. We have used this technique to identify cell surface receptors to a cytokine, tumor necrosis factor-α (TNFα), that are produced as a result of an immune challenge [3]. This approach works well for qualitative studies, but hitherto it has been difficult to quantify the technique so that the numbers per cell of the molecules of interest can be assessed, mainly because of the sensitivity required. One of us has previously used the technique of single-photon counting to quantify fluorescent molecules [4] and showed it to be highly sensitive and reproducible. We report here the application of this technique to the quantitation of fluoresceinated bound antibodies in small biological samples. We have measured the numbers of TNFα receptors appearing on the surface of adipocytes (fat cells) surrounding a rat lymph node at various times after an immune challenge to that node. The change in receptor number confirms and refines our earlier qualitative results and lends support to our hypothesis that adipocytes are locally specialized to modulate the mammalian immune response [5].

KEY WORDS: Tumor necrosis factor-α receptors; adipose tissue; rat; immune response; fluorescent probe.

INTRODUCTION

Fluorescently labeled molecules are widely used in biology as a means of locating and categorizing cells and their constituent molecules. The technique of flow cytometry allows the quantitation of relatively large numbers of cells labeled with a fluorescent marker, but the use of fluorescence to quantitate small biological samples has hitherto been unreliable. Although comparative methods are often used, absolute measurements are not possible with conventional experimental setups. We report here a novel application of single-photon counting to quantify fluorescent molecules in small biological samples.

Our model system is the rat popliteal adipose depot, situated in the hindlimb, and the single lymph node that it contains. Among other things, this lymph node is concerned with counteracting infections in the lower limb, and unless the infection is severe, it can be dealt with at a local level, without provoking a whole-body immune response. Our system therefore models a transient, minor immune challenge, rather than a major disease state. In the response that we study, the lymph node lymphoid tissue interacts strongly with the adipocytes surrounding it, but less strongly with the adipocytes farther away within the same adipose depot and hardly at all with adipocytes from unrelated depots. In particular, it appears that when the lymph node lymphoid cells are subjected to an immune challenge, they produce cytokines and the adipocytes near the node respond to this by producing surface cytokine receptors with a defined time course [3]. One of the functions of the receptors may be to immobi-

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lize cytokines locally so that they are prevented from reaching the rest of the body and provoking an inappropriate immune response [6]. This is particularly likely for the so-called proinflammatory cytokines such as tumor necrosis factor-α (TNFα), which is involved in many deleterious states such as toxic shock syndrome [7]. It is therefore of interest to discover how many TNFα receptors are produced during a minor, local immune response. In addition to our data obtained by single-photon counting, we also present quantitative data obtained by use of a commercially available kit. The two sets of data are broadly comparable but show important differences.

EXPERIMENTAL

Rats were CFHB (Wistar-derived) males, between 8 and 9 weeks old at the time of 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, UK). The left popliteal lymph node, situated behind the "knee," was activated by injecting subcutaneously bacterial lipopolysaccharide (LPS; Sigma, UK), a known elicitor of TNFα production [8], at 1 μg per 100 g body weight into the left lower hindlimb, i.e., distal to the node. This treatment produced no apparent discomfort to the animal, and there was no subsequent discoloration 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 of 60 mg ml^{-1} sodium pentobarbitone (Sagatal, Rhône-Mérieux, Ireland), and the entire popliteal adipose depot, containing its single lymph node, was dissected immediately from each hindlimb, wrapped in foil, and stored at −20°C for up to 3 months. Upon thawing, pieces of adipose tissue were dissected from within 2 mm of the lymph node (near-to-node samples) or from between 5 and 7 mm from the lymph node (far-from-node samples) and washed in Hanks buffered salt solution (HBSS), pH 7.4, then cut into smaller pieces and washed in HBSS + 2% bovine serum albumin (BSA; Sigma). The adipose tissue pieces were incubated with 1 mg ml^{-1} collagenase (Sigma Type II) at 37°C for 30 min to remove extracellular material. Debris was removed by filtration through a 60-μm cell strainer, then the cells were washed a further three times through a 0.45-μm cell strainer. The yield was assessed by hemocytometer count. This procedure gave samples which showed reliable biochemical and physiological differences [5, 6, 9].

For incubation, the samples were processed in a 0.4-μm filter cup (Millipore, UK) in a 24-well plate. Blocking of nonspecific sites was by a 15-min incubation with 100 μl per well of human IgG (Sigma). After washing, the cells were placed in a well containing specific antibody: mouse monoclonal anti-human TNF receptor types I and II (TNF RI and TNF RII), coupled to fluorescein isothiocyanate (FITC) at a ratio of 6 FITC molecules to 1 antibody molecule (R&D Systems, U.K.; catalog numbers FAB225F and FAB226F). The antibodies were used according to the manufacturer's recommended protocol, at a final dilution of 1 in 2. Similar results were obtained with higher dilution factors, but in the bulk of our experiments we used 1:2 to ensure saturating antibody concentrations. The plate was incubated overnight at 25°C in 5% CO_2. The cells were then washed three times, their density was assessed by hemocytometer, and the volumes were adjusted to give 5000 cells per sample.

Single-photon counting was carried out using an Applied Photophysics spectrofluorimeter equipped with a 250-W xenon lamp, an EMI 9813 photomultiplier, and an Ortec 9315 photon counter and standardized using ovalene in a perspex block [4]. The excitation wavelength was 435 nm; preliminary experiments showed that this wavelength gave an optimum signal-to-noise ratio. Emission was scanned in 2-nm steps between 540 and 500 nm. Samples of cells were resuspended by shaking immediately prior to measurement. Each sample was measured twice, and duplicate samples were taken for each point. Data were processed using Microsoft Excel.

For the standard curves, antibody and antigen were added in varying proportions: 100 pg of antigen was added to 0, 1 in 20, 1 in 10, 1 in 5, 1 in 2, and undiluted antibody, and 100 μl of undiluted antibody was added to 10, 20, 40, 60 80, and 100 pg of antigen. Unbound material was separated by passing the samples through a 2 ml Sephadex G50 column eluted with phosphate-buffered saline at pH 7.4. The unbound material was eluted quickly, but the bound complex was retained until the 6.0-ml fraction. These standards were then counted using the fluorimeter. The resulting standard curves are shown in Fig. 1. These confirm that photon counts are linear over the range of concentration of the antigen–antibody complex measured experimentally.

Quantikine assay kits for TNF RI and TNF RII were purchased from R&D Systems, Abingdon, U.K. (catalog Nos. DRT100 and DRT200, respectively), and used according to the manufacturer's recommended protocol, except that cell suspensions were used instead of clear solutions, which is not recommended (see Discussion). The adipocytes were spun down to a minimal volume, then buffer added to give 2000 adipocytes per assay well. Each sample was run in duplicate.

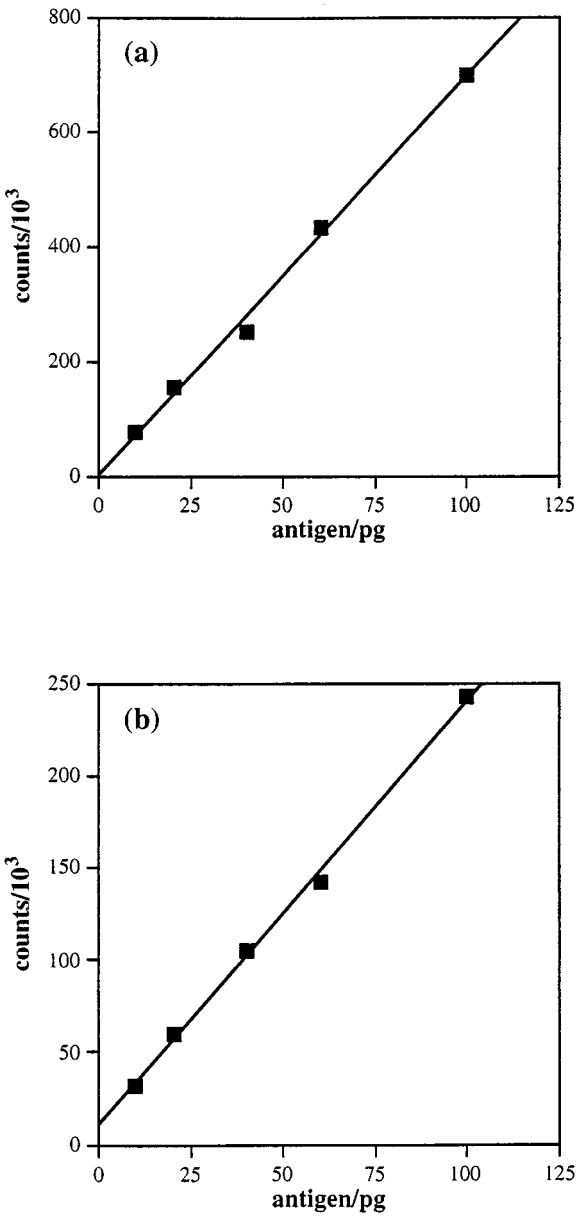

Fig. 1. Standard curves for antibodies to TNFα receptors. (a) Antibody to type I. Slope = 0.143; $r^2 = 0.994$. (b) Antibody to type II. Slope = 0.434; $r^2 = 0.998$.

RESULTS

The data from the photon counting of cells labeled with anti-TNF RI and anti-TNF RII are shown in Tables I and II, respectively. Values are shown as thousands of receptors per cell, calculated using the slopes of the standard curves to derive conversion factors, and M_r values of 55 kD for receptor I and 75 kD for receptor II [10]. For both antibodies there was a peak in the appearance of receptors at 6 h post immune stimulation, and the numbers of receptors declined thereafter, returning to slightly above baseline values by 24 h poststimulation. Numbers of receptors were highest on the samples taken from adipocytes in the stimulated leg, with those cells closest to the lymph nodes producing the most—at least twice the number found on the next highest sample. In adipocytes taken from the unstimulated leg, those closest to the lymph node showed a low response, while those far from a lymph node produced only very few extra receptors. There were consistently more type I than type II receptors at the peak, by a factor of about 2.

The data from the Quantikine analysis of cells labeled with anti-TNF RI and anti-TNF RII are shown in Tables III and IV, respectively. Values are again shown as thousands of receptors per cell. In the case of TNF RI, the highest number of receptors was again reached by about 6 h post stimulation, but the numbers did not subsequently decline, and values remained on a plateau at 24 h. In the case of TNF RII, values continued to increase over the whole 24-h period, although the rate of increase diminished. With both antibodies, the highest numbers of receptors were found on adipocytes taken

Table I. Time Course of Appearance of Type I TNFα Receptors After Immune Stimulation, as Assessed by Single-Photon Counting (Values Are Numbers of Receptors per Cell × 10^{-3})

	Time after stimulation (h)				
Sample	0	1	6	12	24
Stimulated					
Near to node	7.96	23.6	121.1	28.1	8.85
Far from node	7.14	8.75	59.6	8.89	3.23
Unstimulated					
Near to node	5.70	8.88	53.6	3.23	1.33
Far from node	8.83	10.6	14.8	3.30	0.54

Table II. Time Course of Appearance of Type II TNFα Receptors After Immune Stimulation, as Assessed by Single-Photon Counting (Values Are Numbers of Receptors per Cell × 10^{-3})

	Time after stimulation (h)				
Sample	0	1	4	6	24
Stimulated					
Near to node	7.79	5.95	12.9	67.5	13.1
Far from node	5.71	0.17	1.55	30.5	7.34
Unstimulated					
Near to node	4.89	0.80	3.77	13.7	3.63
Far from node	8.02	0.59	1.20	10.9	4.91

Table III. Time Course of Appearance of Type I TNFα Receptors After Immune Stimulation, as Assessed by Quantikine Analysis (Values Are Numbers of Receptors per Cell $\times 10^{-3}$)

	Time after stimulation (h)					
Sample	0	1	4	6	12	24
Stimulated						
Near to node	61	117	273	292	296	316
Far from node	50	70	110	150	109	136
Unstimulated						
Near to node	59	65	86	89	88	124
Far from node	47	58	56	65	69	80

from near a stimulated lymph node, and the lowest numbers were on those far from an unstimulated node. The approximately twofold difference in numbers between TNF RI and TNF RII indicated by single-photon counting was also shown by this technique.

DISCUSSION

The general pattern of these results is as predicted from previously published work [3]. The cells most likely to respond to local signals from an immune stimulated lymph node are those immediately surrounding it, and indeed this sample consistently produced the highest readings. It has been shown that more cells within the same adipose depot can be recruited to respond if the immune challenge is large or repeated [11], and this is consistent with the slight stimulation seen in the number of receptors on cells taken from the same adipose depot but more distant from the signaling lymph node. Some increase in receptors was also seen on cells surrounding the contralateral, unstimulated node, suggesting that, in addition to the local signals passing between stimulated lymphoid cells and surrounding adipocytes, there may also be a systemic "amber alert" that prepares other, unchallenged, nodes for an immune response [12]. As expected, adipocytes distant from an unstimulated node showed little response.

Table IV. Time Course of Appearance of Type II TNFα Receptors After Immune Stimulation, as Assessed by Quantikine Analysis (Values are Numbers of Receptors per Cell $\times 10^{-3}$)

	Time after stimulation (h)					
Sample	0	1	4	6	12	24
Stimulated						
Near to node	30	132	152	186	232	258
Far from node	27	48	56	77	74	153
Unstimulated						
Near to node	30	49	57	50	66	82
Far from node	26	41	39	40	60	69

The main difference between the two sets of results is in the duration of the response. Immunocytochemical studies [3], which are not quantitative, led us to expect at least some receptors to be present at 24 h poststimulation, although not as many as during the peak period of 6–12 h post immune challenge. Both techniques confirmed the presence of receptors at this time, although the single-photon counting data suggested relatively few receptors, whereas the Quantikine data suggested that a large number were still present. We suggest that this difference resides in the different methodologies. Single-photon counting is a highly sensitive and reproducible method for quantifying fluorescent molecules, and we have used it here to assess receptor numbers on small quantities of cells in suspension. In contrast, the Quantikine method, which relies on spectrophotometric detection of a color change, was developed for use on solutions, not cell suspensions (manufacturer's information). It is likely that its application to whole cells could yield erroneous results because of the different physical characteristics of cells and isolated molecules in the reaction wells. In particular, we suggest that the washing procedures might be inadequate, leading to trapping of reagents between the cells stuck in a pellet on the bottom of the reaction wells. This potential source of error could account for both the higher absolute numbers of receptors found and the apparent persistence of a high signal over a longer time period.

Values obtained for the baseline receptor number on adipocytes were about 5000–10,000 per cell for both receptor types. Aggarwal *et al.* reported 2 000 TNFα receptors (type unspecified) per human cervical carcinoma cell [13]. No estimates have been reported for numbers of TNFα receptors on adipocytes, which are very large cells and might be expected to have high baseline receptor numbers.

In conclusion, we report here the application of single-photon counting to the quantitation of cell surface receptors labeled with fluoresceinated antibody. The results give the pattern and time course of receptor distribution expected from our previous work. Although baseline receptor levels are similar, after immune stimulation there appear to be approximately twice as many type I TNFα receptors as type II TNFα receptors on the adipocytes we have studied. We believe these adipocytes to be highly specialized to respond to local signals from lymph node lymphoid cells [3,5,6,9,11,12], and it is possible that the ratio between the two receptor types is func-

tionally important, as they have different roles *in vivo* [3,12]. A broadly similar pattern of results was found using a commercially available kit, but the numbers obtained were higher. However, the commercial kits were not developed for use with whole cells, and the results may therefore be subject to some error. The use of single-photon counting to quantify cell surface receptors represents a simple, straightforward approach to direct measurement, which avoids the complications of other quantitative methods such as Scatchard analysis. It is fairly rapid and, also, uses significantly cheaper consumables than the commercial kits. Nevertheless, the differences observed using the two techniques warrant further investigation.

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