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The inhibitory effect of dexamethasone on lymphocyte adhesion molecule expression and intercellular aggregation is not mediated by lipocortin 1

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SUMMARY

Glucocorticoids exert their anti-inflammatory activity through multiple pathways which include the inhibition of cell adhesion events. The glucocorticoid-induced protein lipocortin 1 (LC1) has reported anti-inflammatory properties and has been proposed as a putative mediator of the anti-inflammatory effects of glucocorticoids. The role of LC1 in mediating the glucocorticoid inhibition of lymphocyte adhesion and cell adhesion molecule (CAM) expression was investigated *in vitro* using a microaggregation assay, flow cytometry and confocal microscopy. Lymphocytes stimulated for 96 h with plastic-bound OKT3 antibody showed significant increases in LFA-1 and CD2 expression. Dexamethasone (DEX; 10^{-6} M) inhibited this increase but the neutralizing anti-LC1 MoAb 1A (5 μg/ml) failed to reverse the DEX effect; neither was purified human LC1 (50×10^{-9} M) able to inhibit CAM expression. The biological activity of the LC1 was confirmed by its ability to suppress monocyte phagocytosis and respiratory burst in response to bovine serum albumin (BSA)–anti-BSA complexes. OKT3 stimulation of cultured mononuclear cells resulted in intercellular aggregation, scored microscopically using a visual index. This aggregation was completely reversed by 10^{-6} M DEX but unaffected by LC1 (50×10^{-9} M). Significant intracellular expression of lymphocyte LC1 was observed using the anti-LC1 MoAb 1B in saponin-permeabilized cells. Distribution of LC1 had a diffuse, cytoplasmic pattern. LC1 expression was reduced following 3 h treatment with 10^{-6} M DEX. These findings indicate that the DEX effects on lymphocyte adhesion and CAM expression are not mediated by LC1. Thus the reported *in vivo* effects of LC1 on leucocyte adhesion and transmigration probably occur through functional/conformation changes of surface CAM, rather than by alteration in expression.

Keywords lipocortin 1 annexin I lymphocyte glucocorticoid cell adhesion

INTRODUCTION

An important facet of the potent anti-inflammatory activity of the glucocorticoids (GC) is their significant effect on lymphocyte adhesion [1–3]. The mechanisms by which GC exert these effects are complex and to certain extent still unknown. Most of the published literature focuses on their ability to regulate gene expression (i.e. their genomic action). However, regulation of post-transcriptional activities have been described and more recently there has been a renewed interest in those mechanisms which act independently of gene regulation (non-genomic mechanisms) [4,5]. In addition, some GC effects are likely to be exerted indirectly through modulation of cytokines or other proteins such as lipocortins. The lipocortins, also known as annexins, are a family of at least 13 calcium and phospholipid-binding proteins. These share a common core structure responsible for the calcium and phospholipid-binding properties, but considerable N-terminal heterogeneity confers the range of biological functions that have been reported within the family [6]. Lipocortin 1 (LC1) has been reported to have anti-inflammatory actions in a variety of animal models of acute inflammation [7,8].

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LC1 levels in circulating leucocytes appear to be under the control of both endogenous and exogenous glucocorticoid hormones. Several studies have indicated that a single bolus administration of anti-inflammatory steroids to human volunteers or experimental animals increases LC1 levels associated with circulating leucocytes [9–11]. Conversely, removal of endogenous corticosterone by means of adrenalectomy or subchronic treatment with a steroid antagonist, mifepristone (RU 38486), reduces cell-associated LC1 levels by >50% [11,12].

A growing weight of evidence now supports a role for endogenous LC1 as a mediator of some of the anti-inflammatory actions of glucocorticoid hormones. Glucocorticoid-dependent LC1 externalization has been demonstrated in monocytes, macrophages and polymorphonuclear neutrophils (PMN) [10,13,14]. Consistent with this, treatment of experimental animals with anti-LC1 antibodies prevented the anti-inflammatory effect of dexamethasone (DEX) in several models of acute inflammation [15,16]. Furthermore, LC1 externalization and release from human PMN occurs during the process of extravasation through the blood vessels, and this is thought to contribute to the feedback regulation of PMN migratory responses [17]. Intravital microscopy studies have revealed that LC1 is capable of inhibiting this transmigratory process [18]. Previous studies have also described an involvement of LC1 in regulation of the inducible form of nitric oxide [19] but failed to find a role for the protein in suppressing IL-1 production by human monocytes [20].

Cell–cell interactions are a critical component of any inflammatory response as they not only facilitate leucocyte migration from the circulation into the surrounding tissues but are also essential for the many contact-dependent immune functions [21]. It has been reported that LC1 can prevent the adhesion of PMN to endothelial surfaces and thus prevent extravasation [22]. We have previously demonstrated that *in vitro* incubation of lymphocytes with GC inhibits adhesion to endothelium and intercellular aggregation via the down-modulation of the adhesion molecules LFA-1 and CD2 [3]. This decrease was accompanied by a fall in the steady-state mRNA level of both genes.

In this current study we investigated *in vitro* the role of LC1 in mediating the inhibitory effect of GC on cell adhesion molecule (CAM) expression and on mononuclear cell (MNC) aggregation. We used two approaches: first, we blocked the action of endogenous LC1 by using the neutralizing MoAb 1A; second, we tested the effects of LC1 directly by using purified placental-derived LC1 in the assays. The results show that, at least in this system, LC1 is not involved in regulating the expression of LFA-1 and CD2. In addition, we also show that LC1 was unable to inhibit intercellular aggregation.

MATERIALS AND METHODS

MNC separation

Peripheral blood from healthy volunteers was collected by venepuncture into heparinized (10 μl/ml 1:1000 preservative-free sodium heparin; Leo Laboratories, Princess Risborough, UK) containers. MNC were separated from diluted blood with an equal volume of Hanks' balanced salt solution (HBSS) by density gradient centrifugation (1250 ***g*** for 20 min) over Ficoll–Hypaque (Lymphoprep; Nycomed, Oslo, Norway) at 20°C, as described [3]. MNC were harvested, washed twice with HBSS at 325 ***g*** for 10 min to eliminate platelets and resuspended in tissue culture medium (TCM) consisting of RPMI 1640 medium (GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS; Sera-Lab, Crawley Down, UK), 0·05 mM sodium hydrogen carbonate (GIBCO), 4 mM L-glutamine (GIBCO) and 10 U/ml penicillin/streptomycin (GIBCO). MNC preparations comprised >90% lymphocytes and <10% monocytes as determined by CD3 and CD14 positivity, respectively. Cell viability was measured in all experiments by trypan blue exclusion and was >95%.

Culture conditions and microaggregation assay

MNC were resuspended at 10^6/ml in TCM and incubated in a moist chamber for 24 h at 37°C, 5% CO_2, either in TCM alone, or in TCM supplemented with water-soluble DEX obtained from Sigma (Poole, UK) at a range of concentrations (10^{-9}–10^{-5} M) during the assessment of dose response. A concentration of 10^{-6} M was used in subsequent experiments. Following steroid incubation, cells were washed with HBSS and cultured at 2×10^6 cells/well in flat-bottomed 24-well plates (Costar, Cambridge, MA) in TCM alone, or TCM and immobilized OKT3 MoAb, in the presence or absence of the neutralizing anti-LC1 MoAb 1A (final concentration 5 μg/ml) [23], a gift from Dr J. Browning (Biogen, Cambridge, MA). Controls included the non neutralizing anti-LC1 MoAb 1B and an irrelevant isotype (IgG1)-matched MoAb P3. In other experiments, purified placental-derived human LC1 (a generous gift from Drs E. Solito and F. Russo-Marie) was added in a concentration range $0{\cdot}5–50\times10^{-9}$ M. A boiled sample to denature and inactivate the LC1 was used as a negative control.

MNC aggregation was assessed by inverted light microscopy following 72 h incubation using an arbitrary visual scale as described by Rothlein & Springer [24]. Briefly, scores range from 0 to 5: 0, no aggregate formation with MNC remaining in culture as single cells; 1, <10% of the cells form aggregates; 2, <50% of the cells aggregate; 3, up to 80% of the cells are in small clusters; 4 and 5, 80–100% of the cells are in large or very large aggregates, respectively. Consistency in visual scoring was achieved by a single observer counting all samples. Results were verified by a second 'blinded' observer.

Immunofluorescence and FACS analysis

MNC cultured as described for the microaggregation assay were stained using a standard single- or double-labelling technique as previously described [25]. Briefly, resuspended MNC, incubated with normal human serum (NHS) to block Fc receptor binding sites, were labelled with MoAbs LFA-1 (CD11a/CD18, ATCC Hybridoma) and OKT11 (CD2; Imperial Cancer Research Fund, London, UK). Washed cells were incubated with FITC-conjugated polyclonal goat anti-mouse immunoglobulin antibody (FITC–GAM; Becton Dickinson, Oxford, UK). Cells were fixed with 1% paraformaldehyde and stored at 4°C for FACS analysis. When cells were double-stained, prior to fixation, they were incubated with normal mouse serum to block any free non-specific binding sites on the previous antibodies and finally stained with a CD3 PE-conjugated MoAb Leu-4 (CD3; Becton Dickinson). Samples were washed twice between each step. Isotype controls included antibodies to irrelevant antigens conjugated with either FITC or PE. Cytofluorometric analysis was performed on a flow cytometer (FACScan; Becton Dickinson). Dead cells and non-lymphoid cells were excluded by setting appropriate forward and 90° light scatter gates. Positive cells were determined by setting a 5% threshold with reference to the relevant negative control. Cell surface antigens were quantified by measuring the mean fluorescence intensity (MFI) expressed in computed units (channel numbers) and compared with reference standard fluorescent

beads (Becton Dickinson). In some experiments, intracellular antigen expression was determined by permeabilizing the MNC with saponin prior to staining with antibodies and appropriate fluorescein conjugates [26].

Confocal microscopy

FITC-stained cells were fixed with a equal volume of 2% paraformaldehyde in PBS and pelleted. Propidium iodide (PI) was added as a nuclear counterstain and cells were mounted onto glass slides and sealed with cover slips. A BioRad (Hemel Hempstead, UK) MRC600 confocal microscope system with 100× objective lens, 25-mW argon ion laser with an excitation line of 488 nm and COMOS analysis software was used to scan the samples and generate photomicrographs.

Phagocytosis assay

Phagocytic uptake of antigen–antibody complexes by monocytes was monitored by a real-time flow cytometric assay. The assay relies upon the intracellular oxidation of an X-rosamine derivative of dihydrodichlorofluorescein (XR-DHDCF) (Molecular Probes Inc., Eugene, OR) which upon excitation at 488 nm emits light at 670 nm, detectable in the FL3 channel of the flow cytometer. The Fc oxyburst RED reagent consists of XR-DHDCF conjugated to bovine serum albumin (BSA) which are subsequently reacted with rabbit polyclonal anti-BSA antibodies to form insoluble complexes in antibody excess [27]. Upon uptake into phagosomes and activation of α nicotinamide adenine dinucleotide phosphate (NAHPH) oxidase, the Fc oxyburst reagent is oxidized to the fluorescent form which can be detected in FL3.

Monocytes were separated from peripheral blood as has already been described. Following washing steps, 250 μl of cells were resuspended at 1×10^6/ml in Kreb's PBS pH 7·4 and incubated for 10 min in a 37°C water bath to equilibrate. A volume of 10 μl of the Fc oxyburst reagent at 3 mg/ml was diluted in 115 μl of Kreb's PBS and equilibrated for 5 min at 37°C. After equilibration, the Fc oxyburst reagent was rapidly added to the cells and immediately applied to the flow cytometer (Becton Dickinson FACScan, set to the high flow-through setting). The monocyte population was acquired and delineated by forward and 90° light scatter characteristics. Fluorescence data were acquired from FL channel 3 in linear mode. Phagocytic responses were quantified using WinMDI software (Dr J. Trotter, Scripps Inst., La Jolla, CA). Two-dimensional density plots of time *versus* FL3-H were gated into 16 equal time slices. Mean FL3-H readings were then plotted for each time slice.

RESULTS

Expression of LC1 in DEX-treated lymphocytes

A combination of confocal microscopy and flow cytometry was used to determine the expression of LC1 in peripheral blood lymphocytes and the effect of 3 h incubation with DEX (1×10^{-6} M). Figure 1A illustrates strong, diffuse cytoplasmic LC1 staining (green) in the majority of lymphocytes, although some cells (leftmost cell) were negative for LC1. Treatment of cells with 1×10^{-6} M DEX (Fig. 1B) resulted in a reduction in LC1 expression. This was confirmed by flow cytometry of saponin-permeabilized cells stained with the anti-LC1 MoAb 1B, which revealed a fall in MFI from 772 ± 107 U in untreated lymphocytes to 570 ± 107 U in cells treated for 3 h with DEX (three observations).

Effects of LC1 on adhesion molecule expression induced by OKT3

In order to determine the contribution of LC1 to the DEX inhibition of CAM expression, MNC were incubated in tissue culture plates coated with OKT3 for 96 h in the presence or absence of 1×10^{-6} M DEX and the neutralizing anti-LC1 MoAb 1A. In experiments on MNC from three different donors, OKT3-stimulated MNC showed significant increases in LFA-1 and CD2 expression. DEX (1×10^{-6} M) inhibited this increase in CAM expression in all donors investigated, although not completely to TCM levels (Fig. 2). MoAb 1A, with neutralizing anti-LC1 activities, showed no significant effect on DEX suppression of LFA-1 or CD2 expression. CAM levels remained similar to those obtained when cells were incubated in the presence of DEX and either an irrelevant isotype control MoAb P3 or the anti-LC1 MoAb 1B, which does not exert neutralizing activity in human cells. In a series of separate experiments using cells from three further healthy donors, purified placental-derived human LC1 was used to determine the direct effect of exogenous human LC1 on CAM expression. Incubation of MNC for 96 h with LC1 at a range of concentrations from 0·5 to 50×10^{-9} M was unable to reproduce the inhibition of LFA-1 and CD2 expression such as was observed with 1×10^{-6} M DEX (Fig. 3).

LC1 inhibits monocyte phagocytosis of antigen–antibody complexes

To confirm the biological activity of the human LC1 preparation, human blood MNC which had been preincubated in the presence or absence of LC1 (50×10^{-9} M) for 1 h at 37°C were incubated with Fc oxyburst reagent to stimulate the endocytotic uptake of these BSA–anti-BSA complexes and activation of NADPH oxidase by monocytes. Figure 4 shows that LC1 preincubation of MNC caused a significant reduction in phagocytosis of antigen–antibody complexes by monocytes incubated with TCM alone. LC1-pretreated MNC which had been in contact with immobilized OKT3 also exhibited a reduced oxidative burst activity. These effects could be completely reversed if the LC1 was denatured by boiling prior to incubation.

Effect of LC1 on the intercellular aggregation of MNC

DEX (1×10^{-6} M) profoundly inhibited intercellular aggregation in four separate experiments. In non-activated cells, this dose of DEX resulted in a 88% reduction in aggregation score whilst 50×10^{-9} M LC1 had no effect on aggregation under these conditions (Fig. 5a). Likewise, in OKT3-treated cells, DEX (1×10^{-6} M) reduced the median aggregation score from 4·1 to 1·3 (68%), whereas LC1 failed to alter aggregation scores over the entire concentration range studied (0·5–50×10^{-9} M) (Fig. 5a). A typical aggregation profile for MNC treated with OKT3 in the presence or absence of DEX and LC1 is shown in Fig. 5b.

DISCUSSION

There is little doubt that some of the most powerful immunosuppressive and anti-inflammatory effects of GC are delivered through the inhibition of adhesion-related processes. Evidence for this comes from *in vitro* studies examining intercellular adhesion, adhesion to endothelium and cell migration [1,3,28]. More importantly, several studies have indicated that this modality of action is operational *in vivo*. Two studies of patients receiving pulse glucocorticoid therapy have reported a decrease in lymphocyte adhesion.

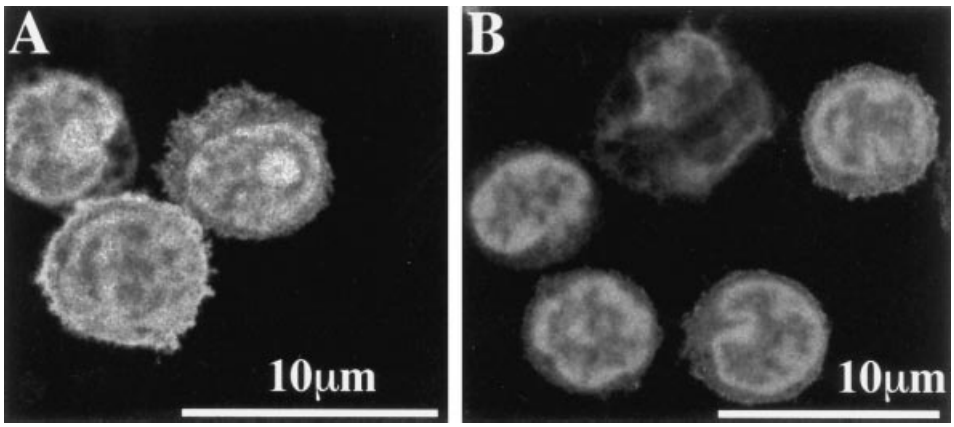

Fig. 1. Confocal micrographs of peripheral blood lymphocytes stained for lipocortin 1 (LC1). (A) Untreated cells, illustrating a diffuse cytoplasmic pattern of intracellular staining. (B) Treatment for 3 h with dexamethasone (DEX; 1×10^{-6} M) resulted in a reduction of expression but no alteration in the staining pattern.

Lymphocytes isolated from multiple sclerosis (MS) patients 3 h following treatment with methylprednisolone showed reduced adhesion to endothelium [29]. Likewise, rheumatoid arthritis patients' blood lymphocytes displayed reduced adhesion to gut lamina propria endothelium 24 h after pulse steroid therapy [30]. Furthermore, direct analysis *ex vivo* of circulating lymphocytes 4 days post-GC therapy in patients with MS demonstrated a decreased expression of LFA-1 and CD2 which was not due to changes in variation of lymphocyte subsets [31]. In addition, analysis of the rheumatoid synovial membrane 24 h after i.v. bolus therapy showed a remarkable reduction of E-selectin and intercellular adhesion molecule-1 (ICAM-1) [32]. A study in healthy individuals, where no inflammation-dependent up-regulation of adhesion molecules would be expected, also revealed a decrease in L-selectin expression by both lymphocytes and neutrophils following i.v. infusions of DEX [33]. This study indicates that the regulation of adhesion molecule expression by GC is part of a physiologic regulatory mechanism. This concept is supported by the study of Tarcic *et al*. who reported that stress-induced increase in endogenous glucocorticoids causes a decreased expression of CD44, LFA-1 and VLA-4 by spleen lymphocytes in BALB/c male mice [34].

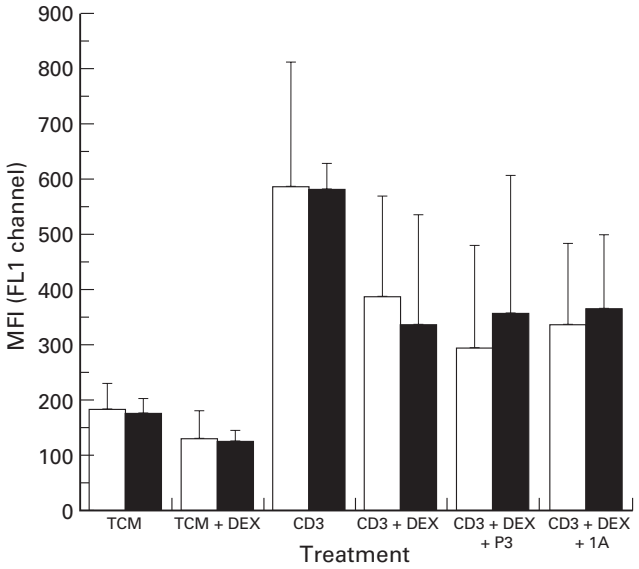

Fig. 2. Effect of the neutralizing anti-lipocortin 1 (LC1) MoAb 1A on the dexamethasone (DEX)-mediated suppression of the lymphocyte adhesion molecules LFA-1 (□) and CD2 (■). Cell surface expression was measured in mean fluorescence intensity units (MFI) + s.d. (three subjects).

The mechanisms by which GC regulate CAM expression/function, as for many other gene products, is likely to be multifactorial. Direct control of gene transcription has been conclusively demonstrated. The binding of the GC–GC receptor complex to glucocorticoid-response elements (GRE) would modulate the interaction of NFAT and AP-1 nuclear proteins with the 5′ flanking regions of the relevant genes [35,36]. GC can also act at post-transcriptional level by regulating translational rates and/or mRNA stability [37]. Finally, GC can exert their effects indirectly by interfering with transcription factors involved in mediating the events which follow cell activation. For example, competition within the nucleus between GC receptors and NFκ-B for binding to the CREB-binding co-activator CBP is thought to be critical to the modulation of subsequent inflammatory response gene activation [38].

The purpose of this study was to investigate whether, *in vitro*, LC1 could regulate CAM expression as another possible indirect modality of action of GC. We have clearly demonstrated that whilst the synthetic glucocorticoid DEX significantly reduced the expression of both adhesion molecules CD2 and LFA-1, these effects were not paralleled by preincubating cells with either neutralizing LC1 antibodies or purified human LC1. Similarly, the latter could not inhibit the intercellular aggregation of activated T lymphocytes. The activity of both reagents was checked in other models, excluding the possibility that these results were due to biologically inert LC1. The activity of anti-LC1 antibodies in neutralizing glucocorticoid activities has been reported *in vivo* [18] and, in agreement with our findings, had no effect on adhesion molecule expression in this system. Likewise, whilst purified human LC1 was shown to be biologically active in the inhibition of monocyte phagocytosis of antigen–antibody complexes (Fig. 3), it had no significant effect on lymphocyte adhesion molecule expression or aggregation.

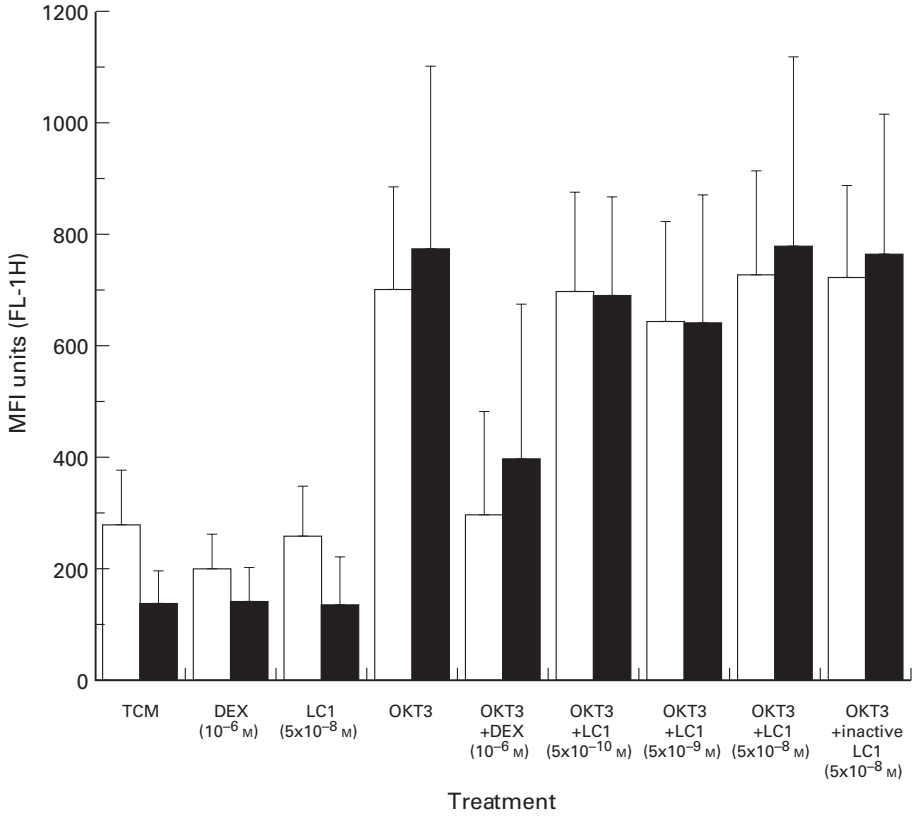

Fig. 3. Comparison of the effects of dexamethasone (DEX) and lipocortin 1 (LC1) on the expression of the adhesion molecules LFA-1 (□) and CD2 (■) in quiescent or CD3-activated lymphocytes. Cell surface expression was measured in mean fluorescence intensity units (MFI) + s.d. (three subjects).

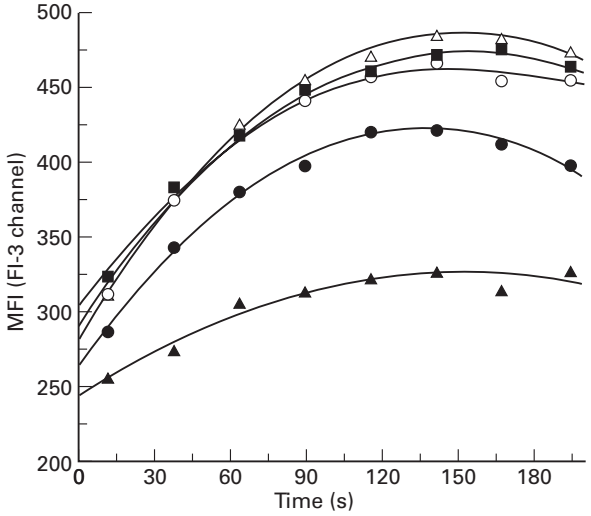

Fig. 4. Representative experiment to show the inhibition of monocyte phagocytosis of bovine serum albumin (BSA)–anti-BSA complexes by human lipocortin 1 (LC1). Uptake of X-rosamine-labelled complexes was measured by flow cytometry in real time and expressed as mean fluorescence intensity units (MFI). Δ, Tissue culture medium (TCM); ▲, TCM + LC1; ○, CD3; ●, CD3 + LC1; ■, CD3 + inact. LC1.

An important aspect to consider in trying to explain these results is the expression of the appropriate LC1 receptors by target cells. It has been proposed that LC1 exerts its pharmacological effects through specific cell surface binding proteins which have been identified on human blood leucocytes [39]. Earlier studies reported differential binding of recombinant human LC1 to human [40] and rodent [41] blood lymphocytes and monocytes, with monocytes exhibiting 100-fold higher LC1 binding than lymphocytes. If indeed LC1 actions are mediated by cell surface binding sites then these results could explain the apparent lack of effect in our *in vitro* system.

However, it is important to point out that these results do not exclude the involvement of LC1 in regulating leucocyte adhesion and migration. It has been proven beyond reasonable doubt, both *in vivo* and *in vitro*, that LC1 is capable of inhibiting the transmigration process [18,22]. Although the mechanisms by which LC1 act in this context are still a matter of speculation, we would propose that LC1 acts by a qualitative (functional modulation) rather than a quantitative regulation of CAM. It is known that, in order to prevent random adhesion in the circulation, CAM are normally expressed on the cell surface in a non-active state (low binding avidity). However, this can be increased dramatically and very rapidly in response to a large number of activating signals including bacterial cell wall components, complement products and a large number of inflammatory mediators [42–45]. Given that GC can mobilize LC1 very rapidly and also that some of its effects on adhesion phenomena are extremely fast, it is conceivable that LC1 may be acting through functional/conformation changes of

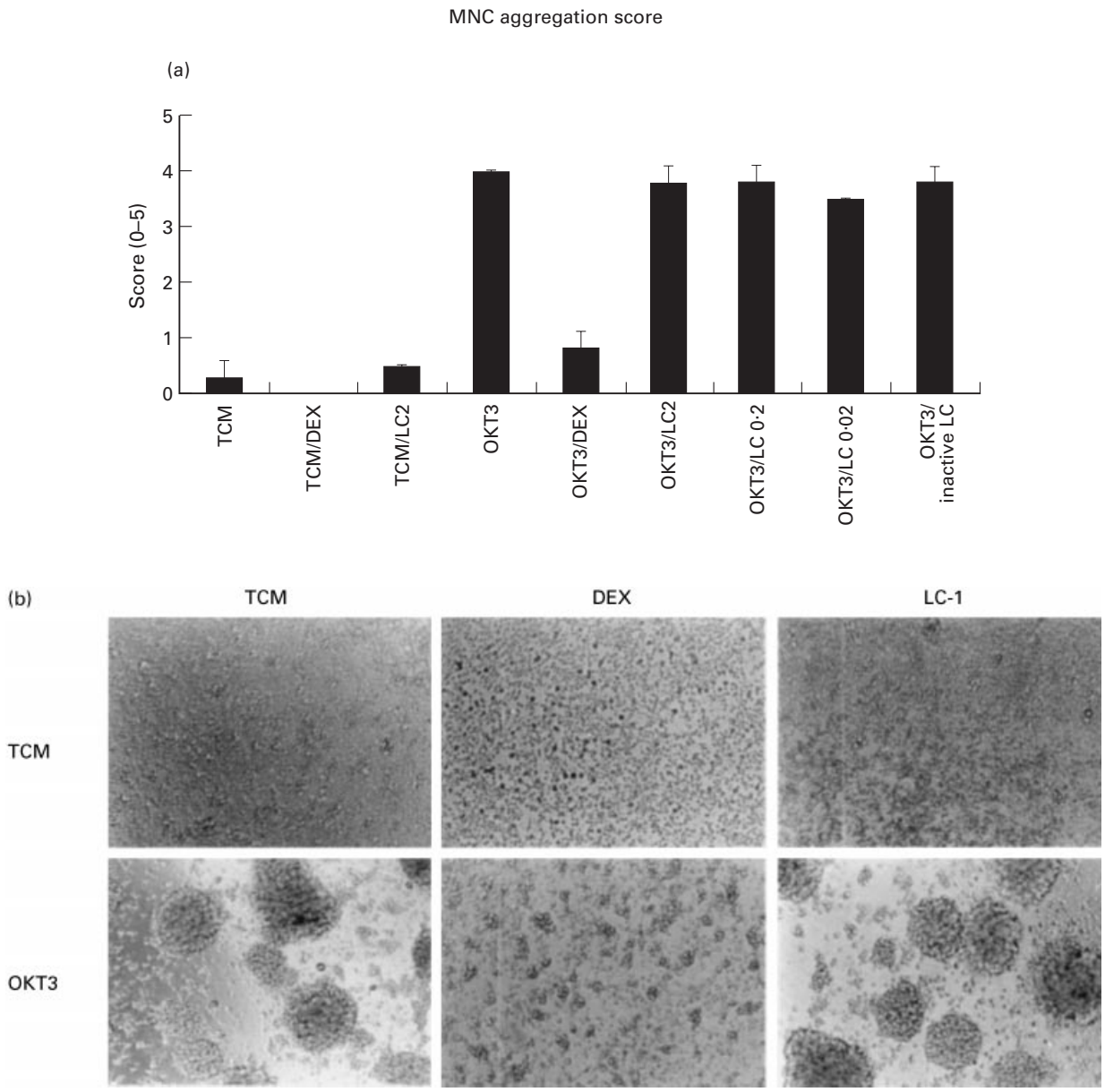

Fig. 5. Effect of dexamethasone (DEX) and lipocortin 1 (LC1) on lymphocyte aggregation following incubation with OKT3. (a) Cellular aggregation scores assessed using an arbitrary visual scale (0–5); results are expressed as median ± s.d. for four experiments. (b) Representative photographs of (i) unstimulated mononuclear cell (MNC), score 0; (ii) MNC stimulated with OKT3, score 4; (iii) MNC stimulated with OKT3 and DEX, score 1; (iv) MNC stimulated with OKT3 and LC1 (50×10^{-9} M), score 4.

surface CAM. There is a precedent for such an activity in that exogenous LC1 inhibits IgG binding to human leucocyte Fcγ receptors and the formation of erythrocyte–antibody (EA) rosettes, but does not alter the level of expression as measured by MoAb binding [46]. Experiments to determine whether a similar mechanism occurs for CAM are currently in progress.

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