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Control of leucocyte function-associated antigen-1-dependent cellular conjugation by divalent cations

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SUMMARY

The control of integrin activation is fundamental to an understanding of the integrin-dependent cellular adhesion thought to be important for a plethora of basic cellular functions. Using a cell–cell conjugation assay the role of divalent cations in leucocyte function-associated antigen-1 (LFA-1)-dependent cellular adhesion was further investigated. The conjugation of interleukin-2 (IL-2)-activated lymphocytes to tumour cells was found to be energy dependent and required the presence of various divalent cations, removal of which decreased the level of conjugation. Increased concentrations of calcium, magnesium and manganese ions resulted in a corresponding increase in levels of conjugation. This increase in conjugation was LFA-1 dependent. Interestingly, when calcium ions were first removed from LFA-1, treatment of lymphocytes with magnesium and manganese ions gave significantly higher levels of conjugation than in the presence of calcium. Using a simple displacement study, calcium ions were shown to displace magnesium ions resulting in decreased conjugation. However, calcium ions were unable to displace manganese ions for binding to LFA-1. That manganese was exerting its effect via an LFA-1-dependent mechanism was confirmed using monoclonal antibodies to CD11a which negated the increased conjugation frequency due to manganese.

INTRODUCTION

The β_2 or leucocyte integrins represent a major subgroup of integrins. Three α-chains (CD11a, b, and c, M_r 175,000, 165,000 and 150,000 respectively) share a common β-chain (CD18, M_r 95,000) giving rise to three distinct adhesion molecules with unique functions; leucocyte function-associated antigen-1 (LFA-1) (CD11a/CD18 heterodimer), Mac-1 (CD11b/CD18), and p150-95 (CD11c/CD18). To date, there are three characterized ligands for LFA-1; namely intracellular adhesion molecule-1 (ICAM-1), -2 and -3.[1]

The relative affinities of the three ICAM for LFA-1 differ markedly, with ICAM-1 having the greatest affinity for its ligand, ICAM-2 and ICAM-3 having similar but lower affinities.[1] The existence of three ligands for LFA-1, the differing tissue distribution, the relative affinities and the varying control of expression by various cytokines indicates specialization for the various aspects of LFA-1-dependent leucocyte interactions. However, in addition to ligand regulation of LFA-1 function, further levels of control exist for this important adhesion pathway.

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It would appear that expression of LFA-1 alone is not sufficient to permit the interaction with ligand to occur as resting lymphocytes do not adhere spontaneously yet express high levels of LFA-1. Interaction of LFA-1 with ICAM is highly regulated, a process which may involve 'activation' of LFA-1 within the membrane. LFA-1, and other integrins, mediate cell adhesion in an energy-dependent manner which requires an intact cytoskeleton.[2] Two main processes may be involved in the activation of LFA-1. Firstly, the avidity of LFA-1 can be modulated by treatment with phorbol ester which causes capping of the antigen.[3] Phorbol 12-myristate 13-acetate (PMA) treatment, therefore, strongly induces LFA-1-dependent cell adhesion.[4] Following treatment with PMA, the co-distribution of LFA-1 and talin has been reported. The cytoskeletal component of the cell would appear to be important in the function of integrins, as disruption of microfilaments with cytochalasins inhibits leucocyte LFA-1 function.[5]

The second form of integrin control is independent of avidity. When molecules such as CD3 are cross-linked with monoclonal antibodies (mAb), intracellular signals are transmitted through the cell, across the membrane to LFA-1 molecules resulting in altered extracellular function[6] (capping may also have a role). Such 'inside-out' signalling is thought to involve the metabolism of inositol phospholipids, thereby activating protein kinase C (PKC), a further mechanism by which PMA may increase LFA-1-dependent functions.[7,8]

The work of two independent research groups, lead by Hogg and Figdor, have done much to elucidate the control of LFA-1 from its 'inactive' to its 'active' state. Two mAb have served as key tools in these studies, namely mAb 24 and NKI-L16.[9,10] These antibodies identify epitopes on the CD11a molecule; however, mAb 24 also binds to CD11b and CD11c.[11,12] Binding of the NKI-L16 monoclonal to its epitope on LFA-1 requires the presence of calcium ions. Resting peripheral blood lymphocytes (PBL) express LFA-1 but lack expression of the L16 epitope.[10,13] However, following stimulation with either PMA or interleukin-2 (IL-2), the L16 epitope is expressed. Although such cells may now aggregate, the expression of the L16 epitope would appear to be prerequisite but not sufficient for LFA-1-dependent function. This is well illustrated by the fact that natural killer (NK) cell clones express high levels of L16 but do not spontaneously aggregate.[14] In contrast, mAb 24 inhibits leucocyte integrin-dependent functions.[15] Expression of the mAb 24 epitope is Mg^{2+} dependent and parallels receptor activity, and may therefore act as an indicator of ligand-bound LFA-1. Furthermore, the binding of ICAM-1 by LFA-1 is dependent upon the presence of divalent cations, in particular Mg^{2+}.[16] Therefore, control of LFA-1 affinity may be achieved by the binding of divalent cations. In the presence of Ca^{2+}, the 24 epitope is not expressed;[11] other workers, however, have shown that Ca^{2+} may synergize with Mg^{2+} giving increased binding.[16] The relative affinity of Mg^{2+} binding is low when compared to Ca^{2+}; however, 1 mM Ca^{2+} does not affect the binding of Mg^{2+}-dependent mAb 24.[17] Unfortunately, the relationship between L16 and 24 expression has not yet been fully dissected. It remains to be determined whether the expression of one epitope induces the other.

The purpose of these studies was to investigate further the effect of divalent cations on LFA-1-dependent conjugation to ICAM-1-expressing target cells rather than to ligand immobilized on plastic. This model is also particularly relevant to effector cell–target cell interactions which may be of clinical importance in immunotherapy as our previous studies have shown that LFA-1-dependent interactions occur between activated lymphocytes and patients' tumour cells. It is possible that this may be involved in tumour destruction in the case of the most successful immunotherapy for any solid human malignancy; that of bacillus Calmette–Guérin therapy for superficial bladder cancer.

MATERIALS AND METHODS

Cell lines and tissue culture

A panel of human transitional cell carcinoma (TCC) cell lines were used (generous gift of Dr J. Masters, Institute of Urology, London, U.K. and Dr P. Perlmann, Stockholm, Sweden). For routine culture, cells were seeded at a density of 5×10^4/ml and cultured in antibiotic-free RPMI-1640 containing 5% fetal calf serum (FCS) complete medium. Cells were recovered by first washing the monolayer with phosphate-buffered saline (PBS) and then trypsinizing using 1 ml trypsin/EDTA solution (0·5 g/l trypsin and 0·2 g/l disodium EDTA) for every 25-cm^2 tissue culture flask. Immediately following detachment of the cells, 10 ml complete medium was added to the cell suspension. Cells were washed once by centrifugation and resuspended in complete medium.

Isolation of peripheral blood mononuclear cells and generation of LAK cells

The isolation of PBMC and their activation using IL-2 was performed as described elsewhere.[18] In brief, this involved separation of lymphocytes using density gradient centrifugation, depletion of adherent monocytes on tissue culture plastic and activation for 6 days using 1000 U/ml recombinant human IL-2.

Dyes

The conjugation assay described was adapted from the manuscript of Caverec and colleagues.[19] Target cells were labelled with the dye hydroethidine (HE) (Molecular Probes, Junction City, OR). After penetration HE is enzymatically oxidized and intercalated into DNA with maximum emission wavelength at 610 nm. HE was prepared as a stock solution of 253 mM in N, N-dimethyl acetamide and stored at 4° protected from the light. A working solution was prepared by diluting the stock solution 1:1000 in PBS. As this concentration was close to saturation, the solution was filtered through a 0·22-μm membrane. Effector cells were labelled with the stain carboxyfluorescein diacetate acetoxymethylester (CFDA.AM) also from Molecular Probes. CFDA.AM is a vital stain and a pH probe (pKa = 6·4). Esterified CFDA.AM produces a fast staining which is enzymatically modified within the cell by esterases with a maximum emission wavelength at 520 nm. The stock solution was 2·5 mM in DMSO and was stored at 4° in the dark. Working solution was prepared by diluting 1:40,000 in PBS. Both dyes can be excited at a common wavelength (488 nm) using a single argon laser.

Staining procedure

Target and effector cells were washed and resuspended at 2×10^6 cells/ml in the working solution of the appropriate dye (HE, 253 μM; CFDA.AM, 62·5 nM). The cells were incubated for 30 min at 37° with agitation every 10 min. Staining was stopped by washing twice in PBS. The final wash was performed in 0·15 M NaCl and cell concentrations were adjusted to yield effector to target ratios of 10:1. Labelled cells were stored at 35° protected from the light.

Quantification of effector/target conjugate formation

Conjugates were formed by mixing 100 μl each of effector and target cells in 0·5-ml Eppendorf tubes, pelleted rapidly by centrifugation and vigorously resuspended using a vortex machine. The formation of conjugates was then assessed. Control for background conjugation was performed by mixing effector and target cells without pelleting.

Flow cytometric analysis of conjugate formation

Flow cytometric analysis was performed using an EPICS-C flow cytometer (Coulter Electronics, Luton, U.K.) operating at 488 nm and 200 mW output. A 70-μm aperture was used with a low differential pressure between sheath and sample. Forward angle light scatter was used to trigger analysis and to gate all other signals from debris smaller than lymphocytes. Analysis was halted when 10^3 target events were recorded. The quantification of conjugate formation based on a fixed number of target cells at a standard effector to target cell ratio was a fundamental element of the procedure. In essence, the number of conjugates formed after 1000 target cells were recorded was expressed as a

percentage of the total number of target cells. Experiments were repeated a minimum of three times.

Antibody-blocking studies

Labelled effector and/or target cells were preincubated with monoclonal antibodies (5 μg/ml) at 20° for 30 min prior to the formation of conjugates as detailed above. Cells were washed prior to the formation of conjugates. Murine monoclonal antibodies to ICAM-1 (RR1/1, IgG1) and ICAM-2 (CBR-IC2, IgG2a) were generously provided by Dr T. A. Springer (The Centre for Blood Research, Harvard Medical School, Boston, MA). Monoclonal antibodies to LFA-1 (CD11a, clone MHM24) were obtained from Dako Ltd (High Wycombe, U.K.). Control reactions were performed with an irrelevant mAb, anti-CD22 (IgG1; SAPU, Carluke, U.K.).

The role of divalent cations

The role of various divalent cations in the formation of conjugates was assessed using calcium, magnesium, and manganese chloride dissolved in 0·15 M NaCl. EGTA and EDTA (both 1 mM in 0·15 M NaCl) were used to chelate divalent cations prior to washing and replacement with the ion of choice. Cells were exposed to divalent cations and chelators of such for 10 seconds. Conjugates were formed, the medium removed and replaced by 0·15 M NaCl containing 0·5% FCS.

Expression of results of conjugation assay

As mentioned above, at a fixed effector: target cell ratio analysis continued until a total of 10^3 target events had been recorded. Of this total, the percentage to which at least one leucocyte conjugated was calculated. Statistical analysis was carried out using the non-parametric Mann–Whitney *U*-test on STATVIEW 512 software.

RESULTS

The formation of stable conjugates between effector and target cells

As various adhesion molecules appear to be involved in the activity of LAK cells against bladder cancer targets,[20] it is reasonable to assume that effector and target cells form stable conjugates in order for cytotoxic events to be initiated. Using a modified flow cytometric-based conjugation assay[19] the efficiency of conjugation to various bladder cancer cell lines was studied.

The percentage of targets which were conjugated to at least one effector cell was expressed as a function of time. Assay before pelleting resulted in low numbers of conjugates, usually less than 2%. Following centrifugation, conjugation was observed to be a rapid and stable event. When cells were subjected to vigorous vortexing, no decrease in conjugation was observed. Our earlier studies have shown a significant correlation between ICAM-1 expression and susceptibility to LAK cells;[21] however, in the present study there was no such correlation ($P > 0·2$) between susceptibility to LAK-mediated cytotoxicity and the ability to form conjugates (Fig. 1). The cell lines RT4 and UMUC-3 conjugated least (15±4% and 12±3%, respectively), higher grade cells forming more conjugates. In apparent contradiction to the cytotoxicity assay,[20] RT112 formed most conjugates (29±8%), suggesting that

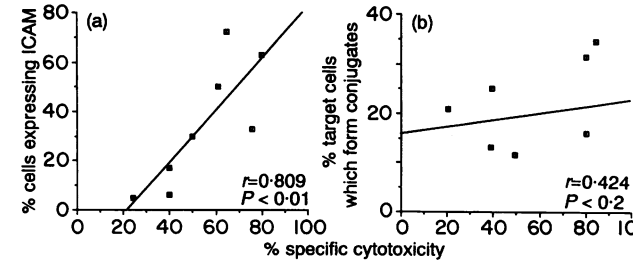

Figure 1. The correlations between (a) constitutive expression of ICAM-1 and -2 and the level of LAK activity against bladder cancer cell lines, and (b) the conjugation frequency and the level of LAK activity. Correlation coefficients (*r*) and the *P*-values are given.

mechanisms other than conjugation are involved in LAK cytolysis.

Freshly isolated PBMC failed to conjugate with any of the bladder cancer cell lines investigated (data not shown) giving levels of conjugation no greater than control levels obtained without centrifugation.

Antibody blockade of conjugate formation

Our previous studies have demonstrated a role for the LFA-1/ICAM interaction in the activity of LAK cells against bladder cancer cells.[20] In the presence of mAb to LFA-1 and ICAM-1, the specific cytotoxicity of LAK cells was reduced. In order to illustrate further the role of these adhesion molecules in LAK activity, adhesion blockade was undertaken using the conjugate assay.

When effector cells were pretreated with mAb to CD11a, their ability to form conjugates with target cells was negated (Fig. 2). The mean level of conjugation for all cell lines in the presence of antibodies to CD11a was reduced by 70·5±23% ($P < 0·01$). However, the level of conjugation of UMUC-3 was not greatly reduced by anti-LFA-1 antibodies (20% reduction). When cells known to express moderate levels of ICAM-1 (MGH-U1, EJ18, SD) were incubated with anti-ICAM-1 antibodies a marked reduction in conjugation was observed. The level of reduction was equivalent to that achieved with anti-CD11a. Conjugation with cell lines RT4 and RT112 (cells which do not express ICAM-1 or -2) was not lessened by antibodies to either ICAM-1 or ICAM-2. Finally, when UMUC-3 was incubated with antibodies to ICAM-1 and ICAM-2 a reduction in the level of conjugation was observed. In the case of anti-ICAM-2 this level of reduction was greater than that achieved with anti-LFA-1.

Conjugation to target cells is an energy-dependent event

The requirement for an active process in conjugation was investigated by using sodium azide to inhibit metabolic processes. When activated lymphocytes were treated with sodium azide conjugation efficiency decreased significantly (Fig. 3). However, similar treatment of the target cells did not affect their ability to form conjugates (data not shown).

The conjugation of effector and target cells is dependent upon divalent cations

Previous work by Dransfield, Marlin and their respective colleagues, has elegantly demonstrated the role of several

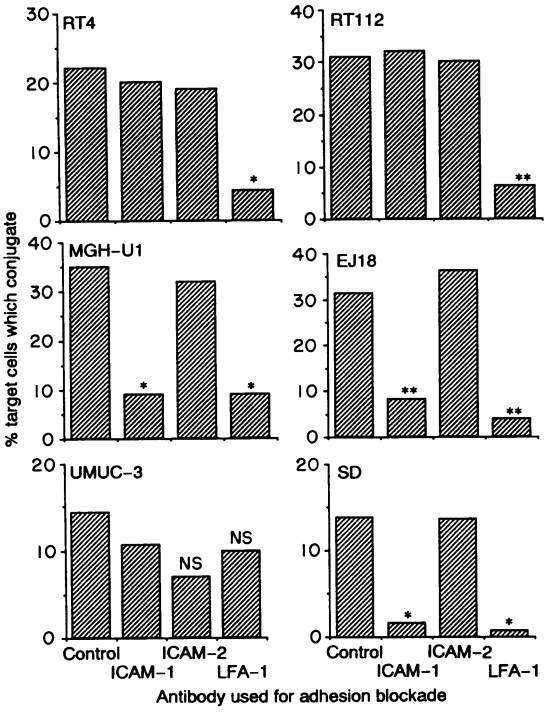

Figure 2. Adhesion blockade decreases conjugate formation. Target cells were incubated with saturating amounts of mAb (ICAM-1 and ICAM-2) while effector cells were incubated with anti-LFA-1 antibodies for 30 min. This incubation was carried out in 0·15 M NaCl. Divalent cations were absent from the reaction although the cells had not been treated with chelating agents. Following washing, conjugates were formed by centrifugation and assessed using flow cytometry. Representative data for several cell lines are shown: $*P<0.01$; $**P<0.002$; NS, not significant.

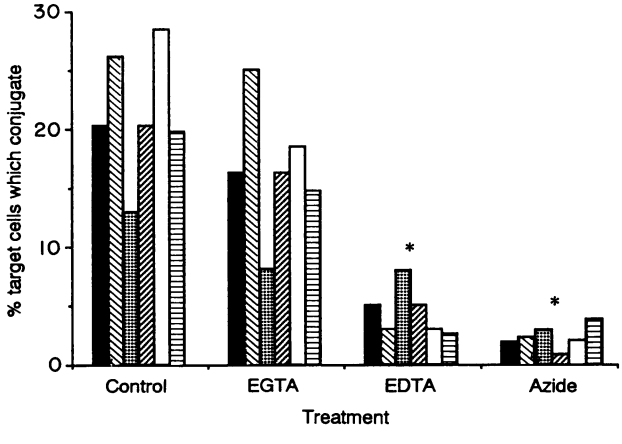

Figure 3. Inhibition of metabolism in the effector cell abolishes conjugation ($P<0.01$). LAK cells were briefly incubated with sodium azide (0·01%) prior to the formation of conjugates and analysis using flow cytometry. Chelation of divalent cations inhibits the ability to conjugate ($P<0.01$). LAK cells were treated briefly with either EGTA or EDTA, both 1 mM. Following washing conjugates were formed. Results for six cell lines are shown: RT4 (■); RT112 (▨); SD (▩); EJ18 (■); MGH-U1 (□); J82 (▤). $*P<0.01$.

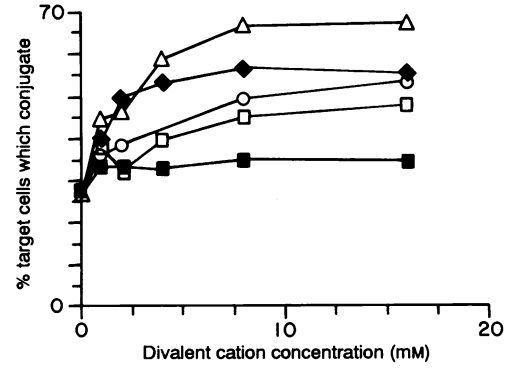

Figure 4. The effect of divalent cations on the formation of conjugates. Effector cells were briefly pretreated with either calcium (□), magnesium (■), manganese (◆), EGTA then magnesium (○) or EGTA then manganese (△), washed and mixed with target cells prior to formation of conjugates. Data shown are for the SD cell line.

divalent cations in the function of LFA-1.[11,16] The role of such cations in the function of LFA-1 as a counter-receptor for ligands on bladder cancer cells was investigated.

When calcium and manganese ions were chelated (EGTA), a small but consistent reduction in the level of conjugation was noted which did not reach significance (Fig. 3). However, removal of all divalent cations using EDTA resulted in levels of conjugation little greater than those achieved following inhibition of metabolism ($P<0.01$).

The role of calcium, magnesium and manganese divalent cations in conjugation

The above studies demonstrate the dependence of conjugation upon the presence of divalent cations. Dransfield and colleagues demonstrated that the binding of mAb 24 to LFA-1 required the presence of magnesium ions[11] and the work of Figdor's group has shown that the expression of the L16 epitope is dependent on the presence of calcium cations.[10] In order to further dissect this phenomenon, the effect of increasing the Mg^{2+} or Mn^{2+} concentration in both the presence and absence of Ca^{2+} was investigated.

When the concentration of calcium, magnesium or manganese in complete medium was increased, the conjugation efficiency of lymphocytes also increased (Fig. 4). Optimal efficiency occurred in the presence of between 4 and 8 mM of each of the cations investigated. When effector lymphocytes were pretreated with manganese cations an increase in binding, greater than in the presence of magnesium or calcium, resulted.

Following chelation of calcium and magnesium ions, the addition of magnesium ions had a dramatic effect upon the percentage of targets which formed conjugates. The level of conjugation in the presence of magnesium, but in the absence of calcium and manganese was greater than in their presence, suggesting competition. Maximal conjugate formation was observed with treatment using manganese, following removal of calcium ions using EGTA.

Calcium ions displace magnesium ions but not manganese ions

The affinity of the cation-binding domain for various divalent cations could reflect the differential response to treatment with

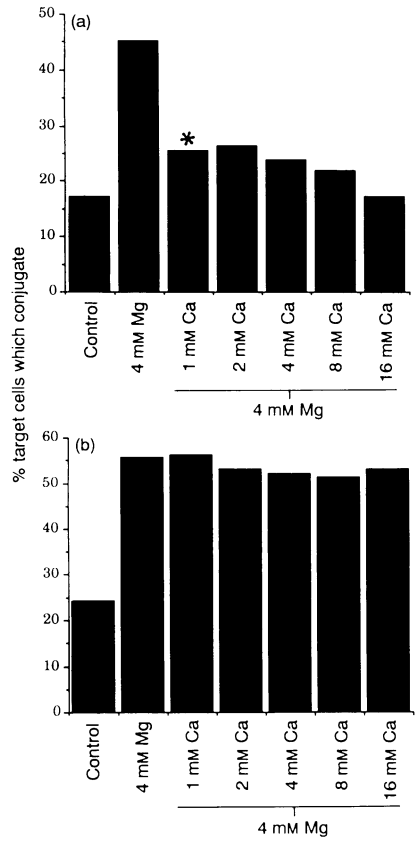

Figure 5. Calcium ions will reverse the effect of magnesium, but not manganese ions. Cells were first pretreated with 1 mM EGTA, washed then briefly exposed to 4 mM magnesium or manganese ions. Following a further wash to remove magnesium or manganese, calcium ions were added at the concentrations indicated, conjugates were formed and analysed. The data shown are those for the RT4 cell line. $*P<0.02$.

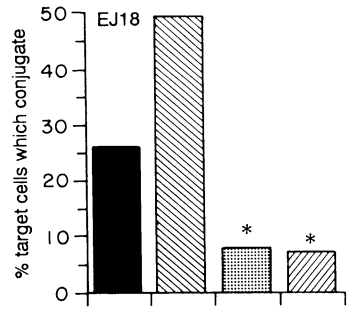

Figure 6. Manganese increases conjugation by an LFA-1-dependent mechanism. Cells were either untreated (■), EGTA then 4 mM manganese (▧), EGTA then 4 mM manganese and sodium azide (▨), EGTA then 4 mM manganese and antibodies to LFA-1 (▨). The data shown are representative for EJ18. $*P<0.005$.

different ion species. The relationship between Mg^{2+} and Ca^{2+} in conjugate formation was investigated with a displacement study. The addition of 1 mM Ca^{2+} to cells pretreated with 4 mM Mg^{2+} resulted in almost complete negation of the increased binding attributed to Mg^{2+} ($P<0.02$) (Fig. 5a). Further increases in calcium ion concentration resulted in a progressive decrease in conjugate formation. This did not appear to be the case for manganese (Fig. 5b).

Manganese increases conjugate formation by an LFA-1-dependent mechanism

The mechanism by which manganese increases conjugate formation was investigated. As CD11a is known to contain cation binding sites, one possibility was that manganese ions were binding directly to LFA-1 thereby leading to conformational changes. Another possibility was that Mn^{2+} was increasing conjugation by an LFA-1-independent mechanism.

When conjugates were formed in the absence of Ca^{2+} and in the presence of Mn^{2+} elevated levels of conjugation were observed. When cells were pretreated with EGTA, then Mn^{2+} and conjugates formed in the presence of sodium azide or anti-CD11a antibodies, a reduced level of conjugation was observed which was significant to the $P<0.005$ level (Fig. 6). This indicated that the increased binding due to manganese was at least partially due to changes in LFA-1.

DISCUSSION

The transient activation of leucocyte integrins provides the necessary intricate control mechanism by which intercellular adhesion may be enabled or disabled. Interactions between IL-2-activated lymphocytes and ICAM expressing tumour target cells were studied using a flow cytometric-based conjugation assay. The purpose of these studies was to investigate whether various divalent cations could alter the function of cell-associated LFA-1 with regard to its interaction with tumour cell-associated ICAM-1 and ICAM-2. Divalent cations differentially increased LFA-1-dependent conjugation. While the effects of calcium and magnesium were reversible, those of manganese were not.

The LFA-1-dependent adhesion of LAK cells to bladder cancer cells is critically energy dependent and requires the presence of divalent cations, in accordance with previous findings.[11,12,22] However, no correlation existed between the percentage of conjugates and LAK activity against targets. Methodologically, the technique employed did not consider the number of effector cells conjugating to a single target cell, a matter for future investigation. Furthermore, it is possible that the target cells possess mechanisms which allow them to resist cytotoxic agents or 'cast-off' adhering killer cells. Deprivation of divalent cations from such cells prevents their forming stable conjugates with target cells. All three cations studied were found to increase the efficiency of conjugation. Furthermore, in the absence of calcium ions, treatment with both Mg^{2+} and Mn^{2+} led to much higher levels of conjugation. With regard to this effect, calcium ions inhibited Mg^{2+}-dependent adhesion, but not

that due to Mn^{2+}. It is possible that manganese is bound with a much higher affinity than the other cations studied, or that irreversible conformational changes take place following the binding of manganese to CD11a. In apparent contrast to these studies, the work of Dransfield *et al.* showed that calcium could compete with both Mg^{2+} and Mn^{2+} for expression of the 24 epitope and for T-cell adhesion.[17] Dransfield's studies also showed that only low levels of binding occurred in the presence of Ca; however, much higher levels are observed under similar conditions between LAK and tumour cells. These studies were performed over a concentration range of divalent cations which was up to 10-fold lower with maximal integrin function observed at 0·25 mM divalent cation. However, our studies were performed with only a brief 'pulse' of cation with the understanding that longer periods of exposure could result in damage to the cell. No decrease in cell viability was seen following treatment with any concentration of divalent cation. It has been suggested that calcium may have a negative regulatory role in LFA-1-dependent functions by inactivating LFA-1.[17] In addition to this, recent evidence shows that the functions of β_1 integrins are also inhibited by calcium ions.[23] Our studies suggest that calcium may not always negate integrin functions. Rather it may maintain LFA-1 in a state capable of intermediate affinity interaction with ligand. Whether differences exist in the LFA-1 molecule on chronically activated cells as compared to classical T cells, or whether these differences are target dependent remains to be determined.

It would seem that LFA-1 exists in a variety of forms: an inactive form, in which state it is unable to interact with ligand; a fully active form possibly expressing both the L16 and 24 epitopes; and intermediate forms which may be capable of limited interaction with ICAM. The reasons for this remain unclear but several possibilities exist. The functions of leucocytes which require adhesion include transmigration, antigen presentation and cell-mediated cytotoxicity. These specialized functions are transient and localized. Furthermore, once adhesion is initiated and completed, it would be beneficial for the leucocyte to detach. Therefore, some regulatory mechanism is required to mediate adhesion and de-adhesion. As for the function of the intermediate forms of LFA-1 these may serve to mark cells which have previously been activated and are therefore of potential use in an inflammatory response (see, for example, Haskard *et al.*[24]).

That divalent cations can regulate LFA-1 activity *in vitro* does not necessarily mean that this occurs *in vivo*. It is possible that these functions mimic the events of 'inside-out' signalling or even cause conformational changes in LFA-1 which are similar to those induced upon binding with ICAM-1. Whether various mAb to integrins can interfere with the binding of divalent cations remains to be determined. Further studies are, therefore, underway to investigate these and other fascinating possibilities.

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