

Mechanisms of lymphocyte adhesion to endothelial cells: studies using a LFA-1-deficient cell line

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SUMMARY

In order to investigate the role of lymphocyte function-associated antigen 1 (LFA-1) in lymphocyte adhesion to endothelial cells (EC), we have studied the adhesion of a LFA-1-deficient lymphoblastoid cell line, ICH-KM, which has < 10% of the cell surface LFA-1 expressed on a normal lymphoblastoid cell line, ICH-BJ. The adhesion of ICH-KM cells to unstimulated EC was $49.9 \pm 8.6\%$ (mean \pm SD) that of ICH-BJ cells. Moreover, phorbol ester-stimulated ICH-KM cells showed a considerably weaker increase in adhesion to unstimulated EC compared with ICH-BJ cells (mean \pm SD increase in percentage adhesion, 3.8 ± 2.3 compared with 18.5 ± 8.0 ; $P < 0.025$). In contrast, there was no significant difference between the enhanced adhesion of ICH-KM cells and ICH-BJ cells to interleukin-1 (IL-1)-stimulated EC. Thus ICH-KM cells showed a 22.7 ± 11.0 (mean \pm SD) increase in percentage adhesion to IL-1-stimulated EC compared with the 24.8 ± 8.5 increase in percentage adhesion of ICH-BJ cells. Anti-LFA-1 monoclonal antibodies had no effect on the enhanced adhesion of ICH-KM and ICH-BJ cells to IL-1-stimulated EC but abolished the differences in adhesion between the two cell lines. The study therefore indicates that although a major part of unstimulated and phorbol ester-stimulated lymphocyte-EC adhesion is dependent upon LFA-1, the enhanced adhesion due to stimulation of EC with IL-1 is not dependent upon this molecule. The data therefore supports the existence of cytokine-inducible LFA-1-independent adhesion molecules for lymphocytes on EC.

INTRODUCTION

The adhesion of lymphocytes to vascular endothelium is the first step in their passage from the blood into inflammatory tissues. As such, the interaction of the lymphocyte with the endothelial cell (EC) has the potential to be of critical importance in controlling the number of lymphocytes in the tissues, which, in turn, may be a rate-limiting factor during an on-going chronic inflammatory response such as rheumatoid synovitis (Cavender *et al.*, 1987b). The analysis of the molecules involved in lymphocyte-EC adhesion is relevant to an understanding of the mechanisms of lymphocyte traffic into inflammatory lesions, and may eventually offer an opportunity to develop anti-inflammatory agents that act by inhibition of lymphocyte migration into inflammatory lesions.

Abbreviations: ^{51}Cr , 51 sodium chromate; EC, endothelial cells; FCS, fetal calf serum; HS, human serum; IFN- γ , interferon-gamma; IL-1, interleukin-1; LFA-1, lymphocyte function-associated antigen 1; LPS, bacterial lipopolysaccharide; P(Bu) $_2$, 4-beta-phorbol-12-13-dibutyrate; TNF, tumour necrosis factor.

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In order to study lymphocyte adhesion to endothelial cells, an *in vitro* model has been constructed that measures the adhesion of 51 sodium chromate (^{51}Cr)-labelled peripheral blood T lymphocytes from normal individuals to endothelial cell (EC) monolayers. It has been demonstrated that T-cell adhesiveness for EC could be directly and rapidly stimulated by phorbol esters (Haskard, Cavender & Ziff, 1986b). Moreover, unstimulated T cells show increased adhesion to EC that have been preincubated with cytokines such as interferon-gamma (IFN- γ) (Yu *et al.*, 1985), interleukin-1 (IL-1) (Cavender *et al.*, 1986), tumour necrosis factor alpha (TNF) (Cavender, Saegusa & Ziff, 1987c) or with bacterial lipopolysaccharide (LPS) (Yu *et al.*, 1986), possibly reflecting a positive feedback mechanism, whereby factors generated by mononuclear cells within inflammatory lesions potentiate the extravasation of lymphocytes into the inflammatory tissue. The effects of simultaneously stimulating lymphocytes with phorbol esters and EC with IL-1, LPS or IFN- γ are additive in their overall effect on lymphocyte-EC adhesion (Haskard *et al.*, 1986b), suggesting that the molecular interactions involved summate in determining the total strength of lymphocyte-EC adhesion. Lymphocytes may adhere to endothelium *in vivo* once a threshold strength of adhesion has been reached.

The lymphocyte function-associated antigen 1 (LFA-1) (CD11a/CD18) is a leucocyte surface antigen belonging to a family of three heterodimer glycoproteins, each with a common 95,000 MW beta chain (CD18) and a unique alpha chain of 180,000 MW (CD11a), 165,000 MW (CD11b) and 150,000 MW (CD11c), respectively (Springer, 1987). LFA-1 has been identified as having an important function in many intercellular interactions involving lymphocytes, including cytotoxic T cell-target cell adhesion (Krensky *et al.*, 1983, 1984; Collins *et al.*, 1984), the spontaneous aggregation of Epstein-Barr virus transformed B-lymphoblastoid cell lines (Mentzer *et al.*, 1985), and phorbol ester-stimulated aggregation of peripheral blood mononuclear cells (Patarroyo *et al.*, 1985; Rothlein & Springer, 1986).

While LFA-1 is present on all peripheral blood lymphocytes, it is not expressed on EC. Experiments using anti-LFA-1 monoclonal antibodies to inhibit T-cell adhesion to EC showed that while a major component of unstimulated adhesion and the adhesion increased by stimulating T cells with phorbol ester was sensitive to anti-LFA-1 monoclonal antibodies, the adhesion increased by stimulating EC with cytokines was relatively resistant to inhibition (Haskard *et al.*, 1986a; Haskard *et al.*, 1987; Cavender *et al.*, 1987c). This suggested that the effect of IL-1, TNF or LPS was to increase the expression on EC of LFA-1-independent adhesion receptors for lymphocytes. It remained possible, however, that the apparent resistance to anti-LFA-1 monoclonal antibodies of lymphocyte adhesion to cytokine-stimulated EC might be due to the existence of functional epitopes on the LFA-1 molecule that are incompletely blocked by available anti-LFA-1 monoclonal antibodies.

The existence of a heritable disease (Arnaout *et al.*, 1984; Beatty *et al.*, 1984; Thompson, Candy & McNeish, 1984; Springer *et al.*, 1984; Kishimoto *et al.*, 1987) in which leucocytes are deficient in the three CD11/CD18 heterodimers which include LFA-1 has allowed the derivation of a LFA-1-deficient B-lymphoblastoid cell line, ICH-KM. This paper describes experiments in which ICH-KM cells have been used to investigate further the role of LFA-1 in lymphocyte EC adhesion.

MATERIALS AND METHODS

Preparation of umbilical vein endothelial cells (EC)

EC were obtained from human umbilical cords by collagenase (Type II, Sigma, Poole, Dorset) digestion as previously described (Cavender *et al.*, 1986). EC were used in the third or fourth passage.

Lymphocytes and cell lines

T cells were isolated from the peripheral blood of normal volunteers by passage of non-adherent mononuclear cells over nylon wool as described previously (Cavender *et al.*, 1986). The B-lymphoblastoid cell lines ICH-BJ and ICH-KM were obtained by Epstein-Barr virus transformation of peripheral blood lymphocytes (Shields *et al.*, 1988). The ICH-BJ cell line was from a normal donor, while ICH-KM cells were derived from a child with CD11/CD18 deficiency, described by Thompson *et al.* (1984). In preliminary experiments the adhesion to EC of ICH-BJ cells was shown to be similar to that of three other lymphoblastoid lines from normal donors.

⁵¹Cr labelling of lymphocytes and lymphoblasts

Lymphoblasts were suspended in 0.2 ml of 15% FCS in RPMI-1640 (assay medium) containing 200 μ Ci Na₂CrO₄ (Amersham, International, Aylesbury, Bucks). Following a 90 min incubation at 37° with intermittent agitation, soluble ⁵¹Cr was removed by washing four times in Hanks' buffered saline. Cell viability was greater than 95% by trypan blue exclusion.

Lymphoblast-EC monolayer adhesion assay

EC at confluence were removed from culture flasks with 0.125% trypsin-EDTA in Puck's saline (Gibco, Paisley, Renfrewshire), centrifuged and resuspended in 15% FCS, 10% human serum (HS) in RPMI-1640 at a concentration of 2×10^5 cells/ml; 0.2 ml aliquots were then cultured overnight or longer in flat-bottomed, gelatin-coated 96-well microtitre plates (Costar, Cambridge, MA). After washing the EC, 5×10^4 Cr-labelled lymphoblasts or 2×10^5 T cells in 0.2 ml assay medium were added per well. Test and control samples were performed in triplicate in each experiment. After incubation for 60 min at 37° in 5% CO₂, the microwells were washed four times with 0.2 ml of warm assay medium to remove non-adherent lymphocytes; 0.2 ml of an aqueous 1% solution of Nonidet P-40 (BDH, Poole, Dorset) was then added to each well and the plate was re-incubated for at least 10 min to lyse the adherent lymphocytes. The percentage lymphocyte-EC adhesion was calculated from the formula:

$$\% \text{ lymphocytes bound} = \frac{\text{c.p.m. in 0.1 ml lysate}}{\text{c.p.m. in 0.1 ml of original lymphocyte suspension}} \times 100.$$

Cytokines

Human recombinant IL-1 beta (IL-1) was from Immunex (Seattle, WA). Human recombinant TNF alpha was a kind gift from Dr B. A. Beutler, University of Texas Health Science Centre at Dallas, TX.

Monoclonal antibodies

MHM-23 (anti-CD18) and MHM-24 (anti-CD11a) (Hildreth *et al.*, 1983) were a kind gift from Professor A. MacMichael, University of Oxford, and were used at saturating concentrations of ascites. W6/32 (anti-HLA class I framework) (Barnstable *et al.*, 1978) was purchased from the European Collection of Animal Cell Cultures and was also used as ascites. Negative control IgG was purchased from Coulter Immunology, Luton, Beds.

Other reagent

The water-soluble phorbol ester 4-beta-phorbol-12-13-dibutyrate [P(Bu)₂] and LPS (*E. coli* serotype 0127:B8) were purchased from Sigma Chemical Co.

Indirect immunofluorescence

Immunofluorescence was performed using an indirect technique as previously described (Pitzalis *et al.*, 1987).

Statistics

Differences between the adhesion of ICH-BJ and ICH-KM cells were evaluated by means of the two-tailed Student's *t*-test.

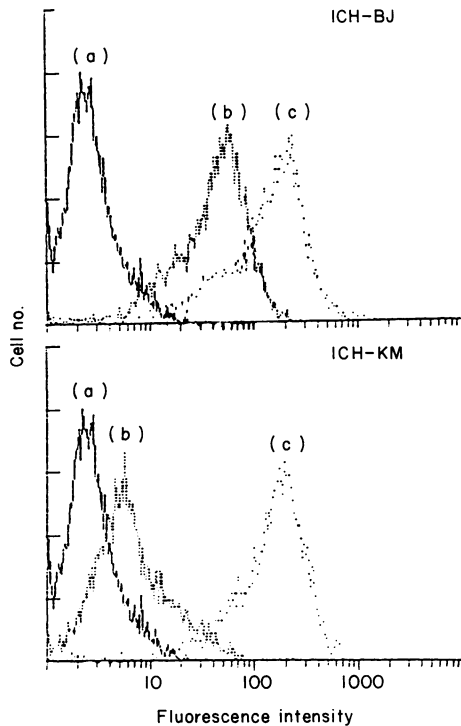


Figure 1. Immunofluorescent staining of ICH-BJ (top) and ICH-KM (bottom) cell lines for (a) irrelevant IgG, (b) anti-CD11a (mAb MHM-24) and (c) HLA class I (mAb W6/32). Note that ICH-KM cells have <10% CD11a of ICH-BJ, compared to normal class I expression. Staining with anti-CD18 (mAb MHM-23) was identical to that of anti-CD11a.

RESULTS

Phenotype analysis of ICH-KM cells

The ICH-KM cell line is an Epstein-Barr virus-transformed B-lymphoblastoid line derived from a patient with CD11/CD18 deficiency. To establish the extent of the LFA-1 deficiency on ICH-KM cells, the binding of anti-CD11a and anti-CD18 mAb was assessed by flow cytometry. As shown in Fig. 1, ICH-KM cells were found to have <10% of the surface CD11 and CD18 seen on control ICH-BJ cells, while the two cell lines expressed a similar quantity of HLA class I molecules, as detected by mAb W6/32 which recognizes an epitope on the HLA class I framework. Neither ICH-KM nor ICH-BJ cell lines stained with mAb specific for CD11b and CD11c (data not shown).

Reduced adhesion of ICH-KM cells to unstimulated EC

Unstimulated ICH-KM cells, which are deficient in cell surface LFA-1, adhered significantly less well to unstimulated EC than unstimulated ICH-BJ control cells. In 11 experiments, the adhesion of ICH-KM cells to unstimulated EC was $49.9\% \pm 8.6\%$ (mean \pm SD) that of ICH-BJ.

Reduced responsiveness of ICH-KM cells to phorbol ester stimulation

Phorbol esters have been shown to increase rapidly lymphocyte-EC adhesion by an action on the lymphocyte rather than

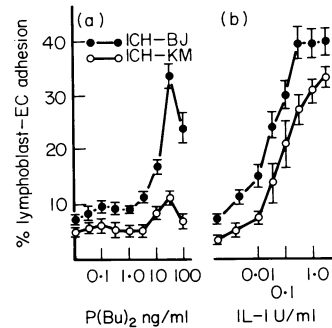


Figure 2. (a) Effect of phorbol ester on the adhesion of ICH-KM and ICH-BJ cells to EC. Varying concentrations of P(Bu)₂ (22 μ l at 10 times final concentration) or medium control were added to the assay wells containing lymphoblasts and EC 30 min before washing off non-adherent lymphoblasts. Values = mean \pm SD. Similar results were obtained in two replicate experiments. (b) Effect of IL-1 stimulation on the adhesion of ICH-KM and ICH-BJ cells to EC. EC were stimulated with varying concentrations of IL-1 for 6 hr and washed before addition of lymphoblasts to the monolayers. Values = mean \pm SD. Similar results were obtained in four replicate experiments.

the EC (Haskard *et al.*, 1986b). When the adhesive response of ICH-KM and ICH-BJ cells to phorbol ester stimulation was titrated in the presence of varying concentrations of phorbol dibutyrate [P(Bu)₂], ICH-KM cells showed a considerably weaker increase in adhesiveness to EC when compared to ICH-BJ cells (Fig. 2a). Thus in three separate experiments, ICH-KM cells showed an increase in percentage adhesion of 3.8 ± 2.3 (mean \pm SD) at an optimal concentration of phorbol ester, compared with the 18.5 ± 8.0 increase in the percentage adhesion of BJ cells ($P < 0.025$).

Normal enhancement of ICH-KM cell adhesion to IL-1, LPS- or TNF-stimulated EC

In contrast to phorbol esters, IL-1 stimulates T-EC adhesion by a mechanism acting on the EC. When the adhesion of ICH-KM and ICH-BJ cells to IL-1-stimulated EC was compared, the two cell lines showed a similar increase in adhesion at all concentrations of IL-1 (Fig. 2b). Thus in five separate experiments, ICH-KM cells showed a 22.7 ± 11.0 (mean \pm SD) increase in percentage adhesion at the optimal concentration of IL-1 (> 5 U/ml), compared with the 24.8 ± 8.5 increase in percentage adhesion of ICH-BJ cells. Similar results were obtained when ICH-KM and ICH-BJ cell adhesion were compared using EC pre-incubated with LPS- or TNF-stimulated EC (data not shown), suggesting the dose-related stimulation by these cytokines of adhesion receptors on EC that do not interact directly with LFA-1.

Effect of anti-LFA-1 mAb on ICH-KM and ICH-BJ cell adhesion to EC

The observation that ICH-KM cells stained weakly with anti-LFA-1 mAb indicated that these cells may have a small amount of functional surface LFA-1. Experiments were therefore performed to investigate the effect of anti-LFA-1 mAb on ICH-KM cell adhesion to EC. As can be seen in Fig. 3, anti-LFA-1 mAb eliminated the small enhancement of ICH-KM adhesion due to phorbol ester, but had no effect on the enhanced adhesion of ICH-KM cells to IL-1-stimulated EC. These findings show

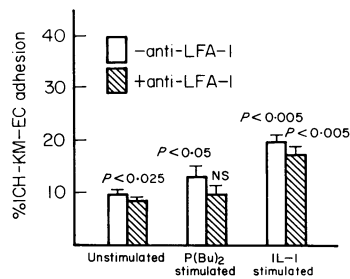


Figure 3. Anti-LFA-1 mAb abolish the small increase in ICH-KM adhesion to EC due to phorbol ester stimulation but do not alter the enhanced adhesion due to IL-1. ⁵¹Cr-radiolabelled ICH-KM cells were pre-incubated for 15 min at 37° with saturating concentrations of mAb MHM-23 (anti-CD18) and mAb MHM-24 (anti-CD11a) (+mAb) or medium control (-mAb). The cells were then transferred to the EC monolayers in the continuous presence of mAb. EC were stimulated with IL-1 (5 U/ml) or medium control for 6 hr and washed before addition of lymphoblasts to the monolayers. P(Bu)₂ (22 μl at 10 times final concentration) or medium control was added to the assay wells containing lymphoblasts and EC 30 min before washing off non-adherent lymphoblasts. Values = mean ± SD. *P* values indicate the significance of values compared to unstimulated adhesion in the absence of mAb. Similar results were obtained in two further experiments.

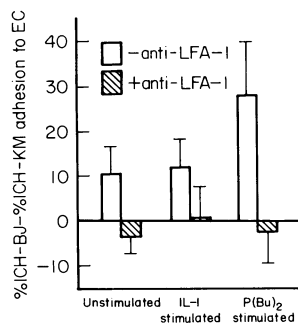


Figure 4. Anti-LFA-1 mAb abolish the differences in adhesion to EC between ICH-BJ and ICH-KM cells. ⁵¹Cr-radiolabelled lymphoblasts were pre-incubated for 15 min at 37° with saturating concentrations of mAb MHM-23 (anti-CD18) and mAb MHM-24 (anti-CD11a) (+mAb). The cells were then transferred to the EC monolayers in the continuous presence of mAb. EC were stimulated with IL-1 (5 U/ml) or medium control for 6 hr and washed before addition of lymphoblasts to the monolayers. P(Bu)₂ (22 μl at 10 times final concentration) or medium control was added to the assay wells containing lymphoblasts and EC 30 min before washing off non-adherent lymphoblasts. The values represent the mean ± SD of three experiments.

that the small amount of phorbol ester-enhanced ICH-KM cell adhesion to EC is attributable to the detectable LFA-1 on their surface.

Anti-LFA-1 mAb abolish the difference in adhesion to EC between ICH-KM and ICH-BJ cells

As can be seen in Fig. 2, when ICH-BJ cells are compared with ICH-KM cells they show greater baseline unstimulated adhesion to EC, a substantially greater increase in adhesion after phorbol ester stimulation, but a similar increase in adhesion to

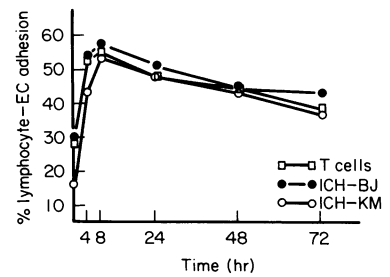


Figure 5. ICH-KM and ICH-BJ cells show similar enhanced adhesion to IL-1-stimulated EC as peripheral blood T cells. EC were pre-incubated for varying lengths of time with IL-1 (10 U/ml) and washed before addition of ⁵¹Cr-labelled lymphoblasts or T cells to the monolayers. Values = means of triplicates. Standard deviations were less than 5% of the mean for each data point.

IL-1-stimulated EC. These differences in adhesion between ICH-KM and ICH-BJ cells were abolished in the presence of anti-LFA-1 mAb (Fig. 4), indicating that adhesive differences between the two cell lines can be accounted for solely by their different expression of LFA-1.

Comparison of the adhesion of ICH-KM and ICH-BJ cells with T cells

To test whether the adhesion of ICH-KM and ICH-BJ cells was similar to that of T cells, an experiment was conducted in which ICH-KM cell, ICH-BJ cell and peripheral blood T-cell adhesion was assayed using EC that had been stimulated with IL-1 for varying times. It can be clearly seen in Fig. 5 that the adhesion of the two lymphoblastoid cell lines closely paralleled that of T cells, with adhesion being maximal 4–8 hr after IL-1 stimulation and plateauing over the ensuing 3 days.

DISCUSSION

The existence of a heritable disease in which leucocytes are deficient in the CD11/CD18 glycoprotein family has allowed the derivation of a lymphoblastoid cell line which expresses < 10% of cell surface LFA-1 found on the normal lymphoblastoid ICH-BJ cell line. It was demonstrated that although ICH-KM cells adhered about 50% as well as ICH-BJ cells to unstimulated EC and showed a substantially weaker increase in adhesion to EC after phorbol ester stimulation, the two cell lines showed an almost precisely similar increase in adhesion to IL-1-stimulated EC. As the adhesion of the two cell lines was identical in the presence of anti-LFA-1 monoclonal antibodies, the observed differences in adhesion between ICH-KM and ICH-BJ cells can be explained by their different expression of LFA-1, without the need to invoke the differential expression of other undefined lymphocyte adhesion molecules.

The apparent resistance to inhibition by anti-LFA-1 monoclonal antibodies of lymphocyte adhesion to cytokine-stimulated EC does not appear therefore to be due to the existence of functional epitopes on the LFA-1 molecule that are incompletely blocked by anti-LFA-1 monoclonal antibodies. These experiments therefore support the previous conclusion (Haskard *et al.*, 1986a) that a major part of baseline unstimulated lymphocyte-EC adhesion is a LFA-1-dependent phenomenon, and that phorbol esters amplify this same adhesion mechanism.

In contrast, the enhanced adhesion due to cytokine stimulation of EC appears to occur by a mechanism not directly related to LFA-1.

The increased adhesiveness of EC due to IL-1 stimulation is prevented by RNA and protein synthesis inhibitors (Cavender *et al.*, 1987a) and is stable to fixation by paraformaldehyde-lysine-periodate (Cavender *et al.*, 1986), suggesting the synthesis and expression of lymphocyte adhesion receptors on the EC membrane. The nature of these putative LFA-1-independent receptors on cytokine-stimulated EC, and their reciprocal ligands on lymphocytes is at present unknown. The relatively prolonged effect of IL-1 on EC adhesiveness for lymphocytes (Fig. 5) compared with the published data for neutrophil adhesion (Bevilacqua *et al.*, 1985) and ELAM-1 expression (Bevilacqua *et al.*, 1987) indicate that ELAM-1 is unlikely to be involved. Similarly a major role for ICAM-1 in IL-1-, TNF- or LPS-stimulated lymphocyte-EC adhesion is unlikely as (i) ICAM-1 is thought to be a receptor for LFA-1 (Marlin & Springer, 1987) and (ii) anti-ICAM-1 mAb combined with anti-LFA-1 mAb are no more inhibitory for IL-1-stimulated T-EC adhesion than anti-LFA-1 mAb alone (D.O. Haskard, unpublished observations). The significance of the observation that IL-1, TNF and LPS enhance ICAM-1 expression on EC (Pober *et al.*, 1986) is therefore unclear.

It is of great interest that individuals such as the patient from whom the ICH-KM cell line was derived, whose leucocytes lack the CD18/CD11 glycoprotein family, develop frequent infections in which inflammatory tissue is deficient in neutrophils. However, lymphocytes have been observed in the inflammatory lesions of these patients, providing *in vivo* support for the hypothesis that lymphocyte migration into inflammatory tissue is not wholly dependent upon expression of LFA-1. It is thus possible that the adhesion system enhanced by IL-1, TNF and LPS, which act on EC, could provide sufficient adhesive support for the extravasation of LFA-1-deficient lymphocytes. Cytokine-enhanced adhesiveness of EC may also partly explain why patients deficient in CD11/CD18 can mount cutaneous hypersensitivity reactions to skin test antigens and do not tend to suffer from severe viral infections (Anderson *et al.*, 1985).

There is increasing interest in the possibility of inhibiting lymphocyte-EC adhesion as a therapeutic approach to the management of inflammatory diseases. As LFA-1 is involved in a large variety of lymphocyte intercellular interactions, its inhibition would not be specific for lymphocyte-EC adhesion. In this light, the nature of the adhesion molecules involved in lymphocyte adhesion to cytokine-stimulated EC deserves further study.

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