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Ruscogenin glycoside (Lm-3) isolated from *Liriope muscari* inhibits lymphocyte adhesion to extracellular matrix

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Abstract

We examined the effects of ruscogenin glycoside (Lm-3), isolated from *Liriope muscari*, on lymphocyte adhesion to extracellular matrix. Adhesion of Jurkat cells activated by anti-CD3 to type I collagen was inhibited by Lm-3 in a concentration- and time-dependent manner. Lm-3 also inhibited the cell attachment to fibronectin and laminin. However, the saponin did not influence anti-CD3-induced cell proliferation and Mn^{2+}-induced adhesion. Protein kinase C activator, phorbol 12,13-dibutyrate, significantly enhanced, while its inhibitor, chlorpromazine, almost completely blocked, the adhesion of anti-CD3-activated Jurkat cells to collagen. Against phorbol 12,13-dibutyrate-activated Jurkat cells, Lm-3 treatment, either before or after activation, significantly inhibited the cell adhesion to collagen. Lm-3 also inhibited the adhesion activated by both anti-CD3 and phorbol 12,13-dibutyrate. Similar inhibition by Lm-3 of the phorbol 12,13-dibutyrate-induced adhesion to collagen was also observed in lymphocytes freshly isolated from mice with contact dermatitis. Furthermore, Lm-3 significantly decreased the leucocyte accumulation in an animal model of experimental pleurisy. These results suggest that the blockade of lymphocyte adhesion to extracellular matrix through interference with the protein kinase C pathway may be one of the mechanisms by which Lm-3 exerts anti-inflammatory activity.

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Introduction

The infiltration of lymphocytes into tissue is an essential component of inflammation. The regulation of cell extravasation is an intricate process involving interactions between adhesion molecules on the leucocyte and specific counter ligands on vascular endothelial cells and extracellular matrix (Springer 1990; Carlos & Harlan 1994; Butcher & Picker 1996). The process of leucocyte migration involves two major steps: extravasation through the vessel wall, and movement through the underlying basement membrane and extracellular matrix (Springer 1994). Extracellular matrix is a complex macromolecular mesh composed of proteoglycans and adhesive glycoproteins such as collagen, laminin and fibronectin (Terranova et al 1986). The adhesion of lymphocytes to extracellular matrix, which is mediated by β1 integrin on lymphocytes, can be activated in response to many stimuli such as cytokines, chemokines and antigen stimulation (Chan et al 1991; Ratner et al 1992; Carr et al 1996). It is not only crucial for the cell motility and retention at inflammatory sites, but also contributes to lymphocyte activation, cytokine production and cell differentiation (Cardarelli et al 1991; Munakata1996). This lymphocyte–extracellular matrix interaction is known to be involved in the pathogenesis of many autoimmune diseases such as rheumatoid arthritis (Pitzalis 1996), allograft rejection (Kupiec-Weglinski & Gorski 1997) and thyroid-associated ophthalmopathy (Bednarczuk et al 1998). Moreover, some monoclonal antibodies and synthetic analogues developed from adhesive molecules have been shown to inhibit inflammatory responses in animal models (Chisholm et al 1993; Hershkoviz et al 1994; Bruck et al 1997; Haworth et al 1998; De Fougerolles et al 2000). These results suggest that intervening in the interaction between lymphocytes and extracellular matrix may be a new strategy for immune suppressive therapies.

At present, immunosuppressants such as glucocorticoids and cytotoxic drugs are usually used for the treatment of inflammatory diseases. However, these drugs are known to cause serious side effects such as suppression of the whole immune system, and sometimes even exacerbation of the disease. To search for more specific immunosuppressants, we have screened a variety of drugs from traditional Chinese medicines for their activity in inhibiting immune response in animal models. As expected, several extracts and their principles from Chinese herbs showed a very promising activity of selectively inhibiting the different phases of immune responses (Xu et al 1991, 1993a–d, 1997; Wang et al 1992; Xu & Xu 1993; Jiang et al 1997; Wu et al 2001). Among them, ruscogenin glycoside (Lm-3), a saponin isolated from *Liriope muscari*, significantly inhibited the delayed-type hypersensitivity and inflammatory response induced by croton oil or xylene in the ear without affecting immune organs (Xu et al 1993d). Furthermore, the saponin and its aglycone, ruscogenin, could improve the liver injury induced immunologically by selectively inducing the dysfunction of liver-infiltrating cells, mainly T lymphocytes, rather than by protecting hepatocyte membranes (Wu et al 2001). These results suggested a new possibility for the therapy of inflammatory diseases by selectively inhibiting the function of activated lymphocytes. To elucidate how this saponin influences the lymphocytes, this study was designed to examine whether the anti-inflammatory activity of Lm-3 is due to its effect on the adhesion of lymphocytes to extracellular matrices.

Materials and Methods

Drugs and reagents

Ruscogenin 1-*O*-[b-D-glucopyranosyl-(1 → 2)]-[b-D-xylopyranosyl-(1 → 3)]-b-D-fucopyranoside (Lm-3) was isolated from the roots of *Liriope muscari* (Decn.) Bailey (Fujian, China). Anti-human CD3 monoclonal antibody was provided by the Department of Immunology, Beijing University Medical Center. The following reagents were purchased: fibronectin, laminin, bovine serum albumin (BSA), cycloheximide, 3-(4,5-dimethyl2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), actinomycin D (Sigma); Type I collagen (Collaborative Biomedical Products, MA); picryl chloride (Tokyo Kassei Industry Co. Ltd, Japan); Bacillus Calmette-Guerin (BCG, Shanghai Biological Product Institute, China); Phorbol 12,13-dibutyrate (Wako Pure Chemical Industries Ltd, Japan); chlorpromazine (Nantong 3rd Pharmaceutical Factory, Jiangsu, China).

Animals

Female ICR mice at 5–6 weeks were obtained from the Experimental Animal House of China Pharmaceutical University (Nanjing, China). They were maintained in plastic cages at 21 ± 2°C with free access to pellet food and water. Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996) and the related ethical regulations of our university. All efforts were made to minimize suffering and to reduce the number of animals used.

Activation of Jurkat cells

Human leukaemia Jurkat cell line was maintained in RPMI-1640 medium supplemented with 100 U mL^{-1} of penicillin, 100 U mL^{-1} of streptomycin and 10% fetal calf serum (FCS) under a humidified 5% (v/v) CO_2 atmosphere at 37°C.

A 96-well microplate was coated with a solution of anti-CD3 monoclonal antibody, 50 l L per well, at 4°C overnight and washed twice with phosphate-buffered saline (PBS). Jurkat cells that had been pre-treated with or without Lm-3 were adjusted to 1×10^6 mL^{-1} and 100 l L was added to each well. After incubation at 37°C for 1 h, the cells were collected and transferred to a plate coated with extracellular matrix for adhesion assay.

Cell proliferation assay

Jurkat cells (1×10^5) were added to anti-CD3 coated wells and co-cultured with Lm-3 at various concentrations for 20 h at 37°C, and then 20 l L of 2 mg mL^{-1} MTT solution were added for further 4 h culture. The supernatant was aspirated carefully and 100 l L of dimethyl sulfoxide was added to dissolve the precipitate. The absorbance was read at 540 nm.

Adhesion assay

A flat-bottom 96-well microplate was coated with 50 l L solution containing type I collagen (50 l g mL^{-1}), fibronectin (5 l g mL^{-1}) or laminin (5 l g mL^{-1}) and left at 4°C overnight. Nonspecific binding was blocked with 0.2% BSA for 2 h at room temperature followed by washing three times with PBS. Then, activated Jurkat cells (1×10^5) suspended in RPMI-1640 were added to each well and incubated at 37°C for 30–60 min. Nonadherent cells were removed by washing three times with RPMI-1640 medium. Then the cells were fixed with methanol–acetone (1:1), and stained with 0.5% crystal violet in 20% methanol. Unbound dye was washed out with distilled water and the plate was air-dried. Bound dye was extracted with 1% sodium dodecyl sulfate. The absorbance of the samples was measured at 592 nm. All assays were run in triplicate, and the results were expressed as percentage of bound cells. The absorbance of 1×10^5 cells, which were fixed and stained without previous washing, was considered as 100% cell adhesion. Specificity of each cell adhesion assay was corroborated using BSA as substratum.

Spleen cells from mice with picryl chloride-induced contact dermatitis

Mice were sensitized by painting 100 l L of 1% picryl chloride in ethanol on the skin of their abdomens. Five

days after sensitization, they were challenged by application of 30 l L picryl chloride in olive oil to the ear. Eighteen hours later, the spleen cells were isolated and used for the adhesion assay.

The spleen cell suspension was prepared as follows. Briefly, spleen was removed sterilely and the cells were dissociated in 5 mL RPMI-1640 medium containing 10% FCS. After centrifugation at 1000 rev min^{-1} for 5 min, 0.17 M Tris (hydroxymethyl aminomethane)–0.75% NH_4Cl solution was added to remove erythrocytes. After washing twice with RPMI-1640 medium, the cells were found to be about 98% viable, as estimated by trypan blue exclusion.

Experimental pleurisy

Female ICR mice were immunized subcutaneously with 0.2 mL emulsion of 10 mg mL^{-1} BCG in saline and incomplete Freund's adjuvant (1:1). Six days later, they were challenged by an injection of 1 mg BCG in 0.1 mL saline into the thoracic cavity. After 24 h, the mice were sacrificed and the chest cavities were opened by midline incision. Exudation in the cavity was collected and rinsed three times with 3 mL RPMI-1640 medium. After removing the erythrocytes using 0.17 M Tris (hydroxymethyl aminomethane)–0.75% NH_4Cl, the cells were suspended in RPMI-1640 medium and counted by trypan blue exclusion.

Statistical analysis

Results were expressed as mean±s.d. Statistical analysis was evaluated by one-way analysis of variance followed by post-hoc Bonferroni test for multiple comparisons, with the level of significance chosen as $P < 0.05$.

Results

Lm-3 inhibited the adhesion of anti-CD3-activated Jurkat cells to extracellular matrices

Jurkat cells pre-treated with or without Lm-3 were activated by anti-CD3. As shown in Figure 1A, activation of the cells by anti-CD3 resulted in an increase in cell counts bound to type I collagen compared with inactivated cells.

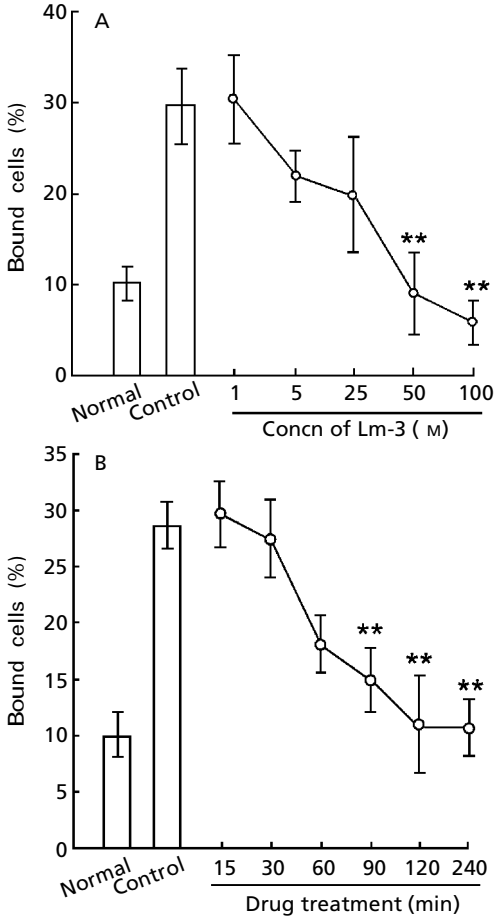

Figure 1 Effect of Lm-3 on the adhesion of Jurkat cells to type I collagen activated by anti-CD3. Jurkat cells (1×10^5) were treated with various concentrations of Lm-3 for 2 h (A) or with 50 l M of Lm-3 for indicated times (B). After washing, they were added to anti-CD3 (10 l g mL^{-1}) pre-coated plates and incubated at 37°C for 1 h. The cells were collected and transferred to type I collagen-coated plates. Cell adhesion was determined as described in Methods. Data are expressed as the mean±s.d. of three separate experiments and each assay was performed in triplicate. One-way analysis of variance revealed a significant effect ($F_{6,14} = 18.006$, $P < 0.0001$ for A; $F_{7,16} = 44.799$, $P < 0.0001$ for B). $^{**}P < 0.01$ vs control (post-hoc Bonferroni test).

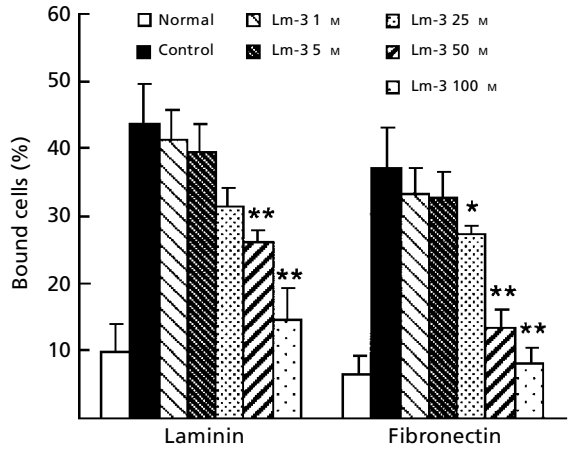

Figure 2 Effects of Lm-3 on the adhesion of Jurkat cells to fibronectin and laminin activated by anti-CD3. Jurkat cells (1×10^5) were treated with Lm-3 for 2 h; after washing, they were added to an anti-CD3 (10 l g mL^{-1}) pre-coated plate. After incubation for 1 h at 37°C, the cells were collected and transferred to a fibronectin (5 l g mL^{-1}) or laminin (5 l g mL^{-1}) pre-coated plate. Cell adhesion was determined as described in Methods. Data are expressed as the mean±s.d. of three separate experiments and each assay was performed in triplicate. One-way analysis of variance revealed a significant effect for fibronectin ($F_{6,14} = 30.602$, $P < 0.0001$) and laminin ($F_{6,14} = 37.872$, $P < 0.0001$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs control (post-hoc Bonferroni test).

Treatment of cells with Lm-3 before activation inhibited the adhesion in a concentration-dependent manner, and 50 l M or higher concentrations of Lm-3 caused a significant inhibition. The kinetics of such inhibition is shown in Figure 1B. Inhibition of T-cell adhesion to type I collagen by Lm-3 was noted after 1 h, and reached a maximum after 2 h. Two other extracellular matrices, fibronectin and laminin, were also used in the cell adhesion assay. As shown in Figure 2, the inhibitory effect of Lm-3 was similar to that in type I collagen.

Lm-3 did not in¯uence the anti-CD3-induced cell proliferation and Mn^{2+}-induced cell adhesion

Jurkat cells were activated by anti-CD3 for 24 h in the presence of various concentrations of Lm-3. The cell proliferation was determined by the MTT method. Almost no influence of Lm-3 on cell proliferation was observed even at the high concentration of 100 l M. In addition, the saponin did not affect cell adhesion to type I collagen induced by another stimulator, Mn^{2+} (data not shown).

Protein kinase C activator and inhibitor regulated the cell adhesion and Lm-3 inhibited cell adhesion induced through the protein kinase C pathway of activation

Jurkat cells were activated with anti-CD3 in the presence of phorbol 12,13-dibutyrate, chlorpromazine, cycloheximide or actinomycin D. As shown in Figure 3, both anti-CD3 and phorbol 12,13-dibutyrate alone caused an increase in the cell adhesion. The combined use of phorbol 12,13-dibutyrate and anti-CD3 significantly enhanced the increase. In contrast, chlorpromazine almost completely blocked the enhancement of adhesion by anti-CD3. However, cycloheximide and actinomycin D did not influence the adhesion activated by anti-CD3.

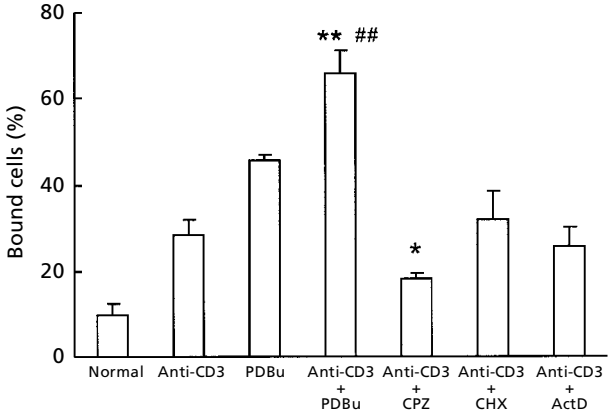

Figure 3 Effect of protein kinase C activator and inhibitor on the adhesion of Jurkat cells to type I collagen activated by anti-CD3. Jurkat cells (1×10^5) were suspended in RPMI-1640 and added to anti-CD3-coated plates in the presence of phorbol 12,13-dibutyrate (PDBu; 100 ng mL^{-1}), chlorpromazine (CPZ; 10 l g mL^{-1}), cycloheximide (CHX; 50 l g mL^{-1}) or actinomycin D (ActD; 1 l g mL^{-1}). After incubation for 1 h at 37°C, the cells were collected and transferred to type I collagen-coated plates. Cell adhesion was determined as described in Methods. Data are expressed as the mean±s.d. of three separate experiments and each assay was performed in triplicate. One-way analysis of variance revealed a significant effect ($F_{6,14} = 88.599$, $P < 0.0001$). *$P < 0.05$, **$P < 0.01$ vs anti-CD3; ##$P < 0.01$ vs phorbol 12,13-dibutyrate (post-hoc Bonferroni test).

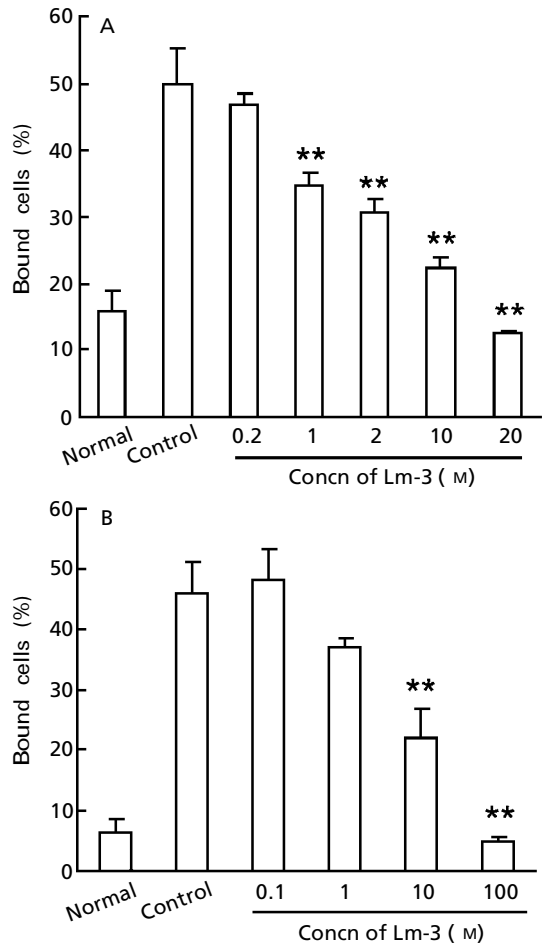

Figure 4 Effect of Lm-3 on the adhesion of Jurkat cells to type I collagen activated by phorbol 12,13-dibutyrate. A. Jurkat cells (1×10^5) were treated with Lm-3 at 37°C for 2 h. After washing three times, they were co-cultured with phorbol 12,13-dibutyrate (100 ng mL^{-1}) for 30 min. B. Jurkat cells were first incubated with phorbol 12,13-dibutyrate (100 ng mL^{-1}) for 30 min. After washing they were treated with Lm-3 for 2 h. After treatment, the cells were added to wells coated with type I collagen and cell adhesion was determined as described in Methods. Data are expressed as the mean±s.d. of three separated experiments and each assay was performed in triplicate. One-way analysis of variance revealed a significant effect ($F_{6,14} = 91.453$, $P < 0.0001$ for A; $F_{5,12} = 77.524$, $P < 0.0001$ for B). **$P < 0.01$ vs control (post-hoc Bonferroni test).

Against the phorbol 12,13-dibutyrate-activated cells, the 2-h pre-treatment with Lm-3 before the activation dose-dependently inhibited the adhesion (Figure 4A). Treatment with Lm-3 for 2 h after phorbol 12,13-dibutyrate activation also showed a similar inhibition (Figure 4B). Similar activity of Lm-3 on the adhesion was also observed in the cells

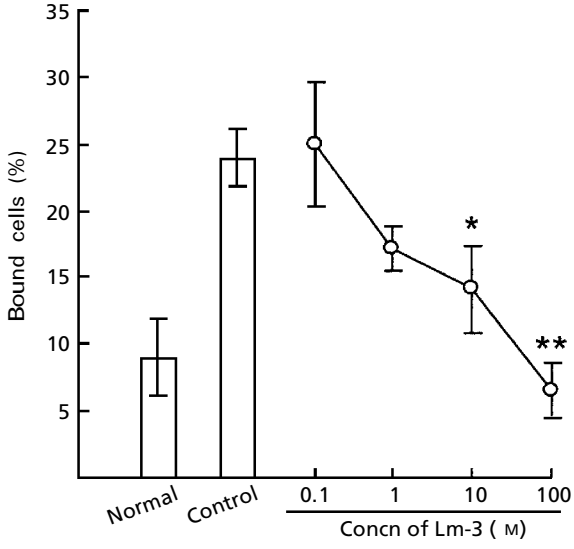

Figure 5 Effect of Lm-3 on the adhesion of spleen cells isolated from mice with contact dermatitis. Mice were sensitized by painting 100 l L of 1% picryl chloride in ethanol on the skin of their abdomens. Five days after the sensitization, they were challenged by application of 30 l L picryl chloride in olive oil to the ear. Eighteen hours later, spleen cells were isolated from the mice and treated with various concentrations of Lm-3 for 2 h. After washing three times, the cells were added to wells coated with type I collagen in the presence of phorbol 12,13-dibutyrate (100 ng mL^{-1}); cell adhesion was determined as described in Methods. Data were expressed as the mean $\pm$ s.d. of three mice and each assay was performed in triplicate. One-way analysis of variance revealed a significant effect ($F_{5,12} = 20.262$, $P < 0.0001$). *$P < 0.05$, **$P < 0.01$ vs control (post-hoc Bonferroni test).

activated by both anti-CD3 and phorbol 12,13-dibutyrate (data not shown).

Lm-3 inhibited the adhesion of spleen cells isolated from mice with contact dermatitis

Spleen cells were isolated from mice with picryl chloride-induced contact dermatitis 18 h after the challenge. The cell adhesion was tested in the presence or absence of Lm-3. As shown in Figure 5, a dose-dependent inhibition of lymphocyte adhesion to type I collagen was observed.

Lm-3 decreased the accumulation of leucocytes at the in-ammation site in experimental pleurisy

Mice were sensitized and challenged with BCG to induce an experimental pleurisy; leucocytes accumulated in the chest cavity and were collected 24 h after the challenge. A great amount of leucocytes was observed in the chest cavity of control mice ($13.64 \pm 9.32 \times 10^6$, n = 9). Compared with this, the three intraperitoneal administrations of Lm-3 (2 h before challenge, and 4 and 10 h after challenge) significantly decreased the leucocyte accumulation. The cell counts were $2.63 \pm 4.44 \times 10^6$ and $2.19 \pm 2.64 \times 10^6$ at the doses of 10 and 20 mg kg^{-1}, respectively ($P < 0.001$ vs control).

Discussion

In our previous studies, we reported that Lm-3 had marked anti-inflammatory activity in animal models (Xu et al 1993d) and improved immunologically induced liver injury by causing the dysfunction of liver-infiltrating T cells (Wu et al 2001). To explore how Lm-3 affected the function of lymphocytes, this study firstly examined the effect of Lm-3 on the adhesion between lymphocytes and extracellular matrix proteins using human leukaemia Jurkat cells. When the cells were activated by anti-CD3, the adhesion to type I collagen increased two-to three-fold. Against this, Lm-3 caused a significant inhibition in a dose- and time-dependent manner (Figure 1). To confirm the effect of Lm-3 on the cell/extracellular matrix interaction, we used two other extracelluar matrix components, fibronectin and laminin, in the adhesion assay, and similar results were obtained (Figure 2). These findings suggest that Lm-3 may interfere with the adhesion process between Jurkat cells and extracellular matrix.

However, the saponin did not influence lymphocyte proliferation induced by anti-CD3. This result excluded the possibility that Lm-3 exerts its inhibition on lymphocyte adhesion by a cytotoxic action. In addition, Lm-3 did not affect the adhesion induced by another stimulus, Mn^{2+}, which can specifically bind the extracellular motif of the adhesion molecule and modulate its function by altering the conformation of the receptor directly (Leitinger et al 2000). This finding implied that Lm-3 might not directly influence the conformational change of adhesion molecules. Since the saponin used for treating cells had been washed out before the adhesion assay, we could suppose that the inhibition of adhesion by Lm-3 was not due to a direct interference with the contact between the adhesion molecules and their ligands.

Upon the activation of cells, the adhesion molecules on lymphocytes transfer from a state of low avidity to a one of high avidity, which leads to the up-regulation of adhesive function of the cells (Smyth et al 1993; Carlos & Harlan 1994). This modulation process involves more than one intracellular signal transduction pathway (Harris et al 2000). Among them, the protein kinase C pathway seems to be essential for the up-regulation of adhesion when T lymphocytes are activated through T cell receptors. In this study, the protein kinase C inhibitor chlorpromazine almost completely blocked, while the protein kinase C activator phorbol 12,13-dibutyrate significantly enhanced, the adhesion of anti-CD3-activated Jurkat cells. However, cycloheximide and actinomycin D showed no influence on adhesion, suggesting that the up-regulation of adhesion is independent of the synthesis of new protein or mRNA. These results might make a linkage to previous reports that the function of adhesion molecules on lymphocytes could

be up-regulated without changes in surface expression (Chan et al 1991).

To explore the relationship between the anti-adhesion activity of Lm-3 and the protein kinase C pathway of cell activation, Lm-3 was used in the adhesion assay induced by phorbol 12,13-dibutyrate alone or together with anti-CD3. The treatment of Jurkat cells with Lm-3 before or after phorbol 12,13-dibutyrate activation dose-dependently inhibited cell adhesion to collagen (Figure 4). A similar result was obtained in cells activated by both phorbol 12,13-dibutyrate and anti-CD3 (data not shown). It should be noted that cells activated by phorbol 12,13-dibutyrate alone seemed to be more susceptible to Lm-3 than those activated by anti-CD3, as 1 l M of Lm-3 in the former (Figure 4A) but more than 50 l M in the latter (Figure 1A) was needed to cause a significant inhibition. As mentioned above, phorbol 12,13-dibutyrate could activate intracellular protein kinase C directly, while anti-CD3 induced adhesion through the activation of many intracellular signals including protein kinase C activation after binding to the receptor on the T cell surface. Our finding suggested that the protein kinase C pathway might be easily influenced by the saponin. The efficacy shown in Figure 4B also indicated that Lm-3 did not affect the activation of protein kinase C but might block the intracellular signalling downstream of the protein kinase C activation. However, further study on the detailed mechanism is in progress.

The aforementioned in-vitro studies supported the finding that some stimuli may up-regulate the adhesion of Jurkat cells to the extracellular matrix (Wilkins et al 1991) and suggested that interference with the lymphocyte adhesion is involved in the anti-inflammatory activity of Lm-3. As many reports have indicated, the localization of lymphocytes at an inflammation site is a carefully coordinated multi-step process in which the adhesion of lymphocytes to extracellular matrix plays an important role (Springer 1990; Carlos & Harlan 1994; Butcher & Picker 1996). To confirm the implication of the inhibition of cell adhesion by Lm-3 in the actual immunological inflammation, freshly isolated spleen cells from mice with picryl chloride-induced contact dermatitis were applied to the adhesion assay. Against this model, which is mediated by CD^{4+} T lymphocytes, our previous study has demonstrated the efficacy of Lm-3 when given during the effector phase (Xu et al 1993d). This study gave a further explanation for the efficacy, namely that the saponin dose-dependently inhibited adhesion to type I collagen of spleen cells isolated from mice 18 h after challenge with picryl chloride (Figure 5), when the adhesion of spleen cells to extracellular matrix reached maximum (data not shown).

Furthermore, an experimental pleurisy was used to confirm the anti-adhesion effect of Lm-3 in-vivo. Pleurisy is known to be a delayed inflammation composed chiefly of lymphocytes 3 days after induction (in comparison with the dominant granulocytes in the initial stage) and adhesion molecules are involved in the cell accumulation (Widstrom & Nilsson 1982; Menezes-de-Lima-Júnior & Henriques 1997). As a result, Lm-3 (10 and 20 mg kg^{-1}), administered in the effector phase, significantly decreased cell accumulation in the chest cavity. This finding allows a linkage of the in-vitro finding of inhibition of cell adhesion by Lm-3 to the blocking of leucocyte infiltration into the inflammation site.

Since adhesion of lymphocytes increased only after they were activated by various stimuli, this suggests that Lm-3 exerts its inhibitory action on lymphocyte adhesion in an immune response but not on normal cells. This finding further supported our previous data that Lm-3 inhibited inflammation without affecting immune organs and selectively caused the dysfunction of liver-infiltrating lymphocytes in the immunologically induced liver injury (Xu et al 1993d; Wu et al 2001). In addition, the saponin affected neither lymphocyte viability nor apoptosis; nor did it affect the release of NO and H_2O_2 by macrophages (data not shown). Moreover, Lm-3 could inhibit anti-CD3-induced cell adhesion but not lymphocyte proliferation. These characteristics are quite different from those of immunosuppressants such as glucocorticoids or cytotoxic drugs.

In conclusion, this study confirmed the inhibition of lymphocyte adhesion to extracellular matrix by Lm-3 through interference with the protein kinase C pathway that may be involved in its anti-inflammatory activity. Although the underlying mechanism needs to be elucidated, this study has raised the possibility of selectively blocking certain functions of the cells without affecting other functions.

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