Activation Pattern of Langerhans Cells in the Afferent and Efferent Phases of Contact Hypersensitivity

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Langerhans cells are MHC class II (Ia) positive antigen-presenting cells that play a crucial role in the induction of contact hypersensitivity (CHS). The topical application of a hapten modifies the cell surface moieties of Langerhans cells, and activates Langerhans cells to increase their size and Ia intensity. The haptenated and activated Langerhans cells emigrate from the epidermis and thus the *in situ* density of Langerhans cells usually decreases during 24–48 h after the hapten application in CHS. To determine whether the early activation pattern of Langerhans cells is different between the afferent phase and the efferent phase of CHS, we compared the density and morphologic changes of Langerhans cells in CHS to trinitrochlorobenzene using nonsensitized and sensitized mice. We found that the application of a hapten induces more significant enlargement of Langerhans cell size in the afferent phase than in the efferent phase, whereas the reduction of Langerhans cell density is more marked in the efferent than in the afferent phase of CHS. Moreover, topical immunosuppressive drugs inhibit the *in situ* activation of Langerhans cells. *Key words: contact hypersensitivity/cyclosporinee/FK-506/Langerhans cells. Journal of Investigative Dermatology Symposium Proceedings 4:164–168, 1999*

Contact hypersensitivity (CHS) represents a highly specific, long-lived, and reproducible delayed-type hypersensitivity that is a very useful experimental model or prototype to analyse T cell-mediated cutaneous inflammation (Ptak *et al*, 1980; Katz, 1993). Topical application of an antigenic hapten to skin can sensitize the individual (afferent or inductive phase), and the epicutaneous readministration of the identical hapten induces hapten-specific dermatitis of delayed-onset at 24–48 h (efferent or elicitation phase) (Botham *et al*, 1987; Kalish, 1991; Katz, 1993).

In the afferent phase of CHS, there is ample evidence that Langerhans cells play a pivotal role in compelling the sensitization (Toews *et al*, 1980; Kripke and McClendon, 1986; Kripke *et al*, 1990). Langerhans cells are Ia-positive epidermal dendritic cells that act as potent antigen-presenting or accessory cells for primary and secondary T cell-dependent immune responses (Silberberg *et al*, 1974; Stingl *et al*, 1978; Toews *et al*, 1980; Inaba *et al*, 1986; Kalish, 1991; Katz, 1993; Steinman *et al*, 1995). The Langerhans cell-containing epidermal cells can sensitize mice to haptens (Tamaki *et al*, 1981; Furue and Tamaki, 1985; Sullivan *et al*, 1985), and Langerhans cells can directly induce antigen-specific T cells (Stingl *et al*, 1978; Hauser, 1990). Resident epidermal Langerhans cells are immature antigen-presenting cells (APC), but mature into potent APC after capturing antigen. The topical application of a hapten modifies the cell surface moieties of Langerhans cells, and activates Langerhans cells to increase their size and Ia intensity (Aiba *et al*, 1984; Aiba and Katz, 1990). The enlargement of Langerhans cell size *in situ* seems to be closely associated with their functional maturation because quite similar enlargement occurs in cultured Langerhans cells that acquire very potent immunostimulatory function *in vitro* (Schuler and Steinman, 1985; Shimada *et al*, 1987; Witmer-Pack *et al*, 1988; Kawamura and Furue, 1995). The haptenated and activated Langerhans cells emigrate from the epidermis to the dermis and then into the regional lymph nodes where they can sensitize T cells (Kripke *et al*, 1990; Katz, 1993). The emigration of stimulated Langerhans cells causes a decrease of *in situ* density of Langerhans cells in the allergic and occasionally irritant CHS in mice and humans (Aiba *et al*, 1984; Botham *et al*, 1987; Marks *et al*, 1987; Hanau *et al*, 1989; Fujita *et al*, 1990; Willis *et al*, 1990). In skin transplant experiments, Langerhans cells in epidermal sheets from allograft, isograft, and explants dramatically increase not only in size but also in terms of their immunostimulatory function, and their numbers are markedly decreased (Larsen *et al*, 1990), indicating that there is a close relationship between the morphologic alteration and the emigration of Langerhans cells.

Although the *in situ* activation pattern (enlargement and decreased density) of Langerhans cells is well documented in the afferent phase and efferent phase of CHS, little is known about whether the dynamic activation pattern of Langerhans cells is different between the afferent phase and the efferent phase of CHS. We have compared the density and morphologic changes of Langerhans cells *in situ* in the afferent and efferent phases of CHS to trinitrochlorobenzene (TNCB) using nonsensitized and sensitized mice. We demonstrate that the activation pattern (decrease of density or increase of size) of Langerhans cells is differentially regulated in the afferent phase and efferent phase of CHS, and that topical immunosuppressive drugs inhibit the *in situ* activation of Langerhans cells.

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Abbreviations: CHS, contact hypersensitivity; CS, cyclosporinee; DEX, dexamethasone; TNCB, trinitrochlorobenzene.

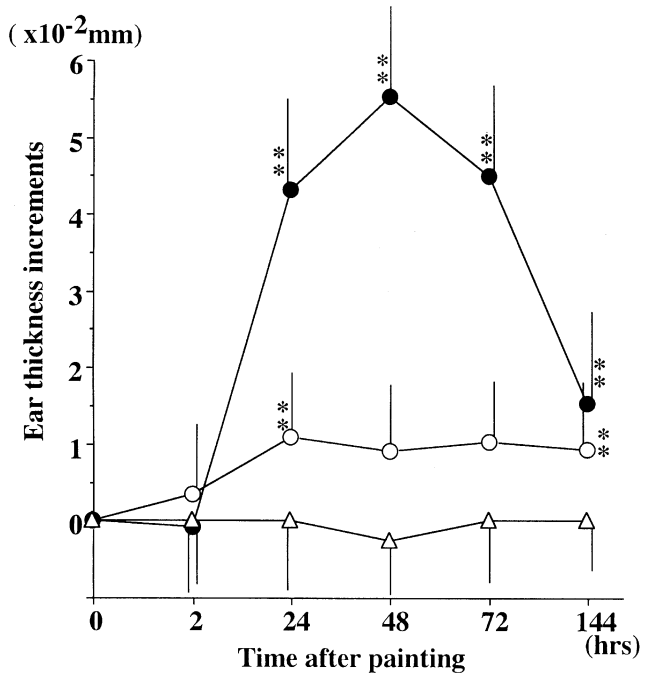

Figure 1. Time course of ear swelling after 1% TNCB painting in the unsensitized (afferent phase) and sensitized (efferent phase) mice. Marked ear swelling was observed in the sensitized mice when compared with the control olive oil application. Slight ear swelling was evident in the unsensitized mice. △, Olive oil only (n = 8); ○, TNCB painting (unsensitized group) (n = 17); ●, TNCB painting (sensitized group) (n = 22). Data show mean ± SD. **p < 0.01.

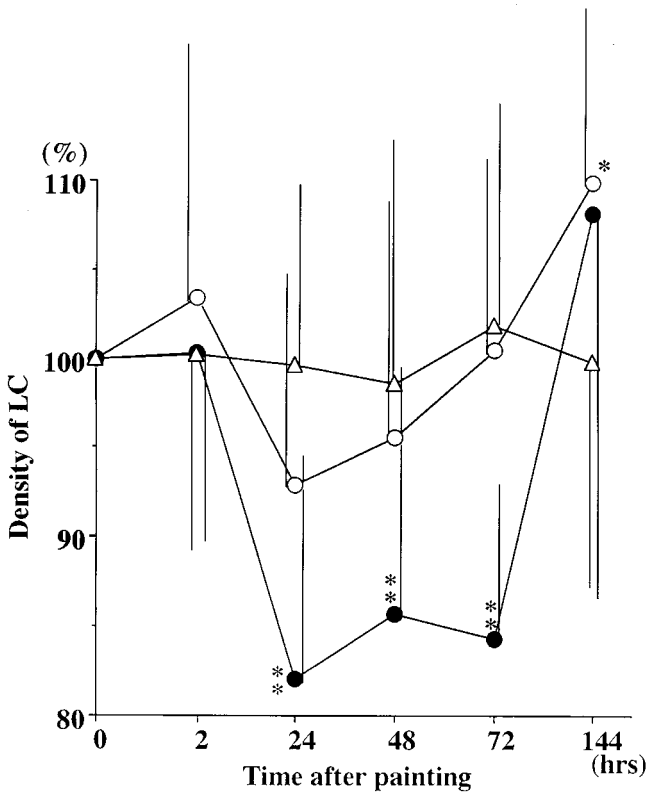

Figure 2. Time course of Langerhans cell density after 1% TNCB painting in the unsensitized and sensitized mice. A significant decrease of Langerhans cell density was detected at 24–72 h in the sensitized mice. In the unsensitized mice the decrease of Langerhans cell density was very weak and was not significant. △, Olive oil only (n = 20); ○, TNCB painting (unsensitized group) (n = 16); ●, TNCB painting (sensitized group) (n = 16). Data show mean ± SD. *p < 0.05; **p < 0.01.

MATERIALS AND METHODS

Animals Female C3H/HeN mice, 8–12 wk old, were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan).

Reagents 2,4,6-Trinitrochlorobenzene (TNCB) was obtained from ICN Pharmaceuticals (Plainview, NY). Anti-I-Ak (clone,11–5.2: mouse IgG2b) and control mouse IgG2b antibodies were purchased from Pharmingen (San Diego, CA). FITC-conjugated goat antimouse IgG was obtained from Cappel (Durham, NL). Dexamethasone (DEX) was purchased from Sigma (St Louis, MO). Cyclosporinee A (CS) was obtained from Sandoz (Tokyo, Japan). FK-506 was obtained from Fujisawa (Osaka, Japan).

Sensitization and elicitation of CHS C3H mice were sensitized by epicutaneous application of 100 μl of 7% TNCB in a 4:1 acetone:olive oil mixture on the shaved abdomen as previously described (Furue and Tamaki, 1985). Six days later, 20 μl of 1% TNCB in olive oil was topically applied on the ears of sensitized and nonsensitized mice. The olive oil application served as a solvent control. Ear thickness was quantitated with a micrometer (Mitsutoyo, Tokyo, Japan) at 2, 24, 48, 72, and 144 h after challenge, and compared with the thickness of the same ear assessed prior to challenge. After thickness measurement, the epidermal sheets were prepared from the ears at the same time point and immunolabeled with anti-I-Ak antibody. From five to 15 mice were used in each group. Experiments were performed at least three times.

We chose 20 μl of 1% TNCB in olive oil as the elicitation dose in order to directly compare the afferent and efferent phases of CHS, because a suitable minimum concentration of TNCB to actively induce both sensitization and elicitation of CHS was 1% in C3H mice (data not shown), and the olive oil diluent had no effect on the CHS response *in vivo*. Thus, using this experimental protocol, we could simultaneously examine the afferent phase (using nonsensitized mice) and the efferent phase (sensitized mice) of CHS for the same amounts of the hapten.

Separation and immunostaining of epidermal sheet The ears were first mechanically divided into dorsal and ventral sides of cutaneous sheets. The epidermis was then separated from the dermis after incubation with 3.8% ammonium thiocyanate (Sigma). After the fixation with acetone at –20°C for 20 min, the epidermal sheets were incubated with anti-I-Ak or control mouse IgG2b antibodies overnight at 37°C, washed with phosphate-buffered saline, and further incubated with FITC-conjugated goat antimouse IgG for 2 h at 37°C. The stained sheets were observed by Zeiss immunofluorescence microscopy. Control isotype matched mouse IgG2b always yielded negative results.

Density and relative size of Ia^+ Langerhans cell in the epidermal sheets The number of Ia^+ Langerhans cells was enumerated by using a rectangular ocular grid at a magnification of 400×. At least 10 areas of each epidermal sheet were randomly chosen and the mean number of stained cells per mm^2 was determined in each experimental group. The density of Langerhans cells was expressed as a relative percentage compared with the Langerhans cell density of normal control mice standardized as 100%. The immunolabelled Langerhans cells were then photographed at the magnification of 400×. The images of Ia^+ Langerhans cells were computerized by photoscanning. The mean size of Langerhans cells in untreated normal mice was arbitrarily expressed as 100, and the relative size of Langerhans cells was calculated in each experimental group using the computer program "NIH Image".

Effects of topical immunosuppressive drugs The immunosuppressive activity of DEX, CS, or FK-506 had been confirmed as described previously (Furue and Ishibashi, 1991; Furue *et al*, 1994). DEX, CS, and FK-506 were dissolved in 2:1 dimethlsulfoxide:ethanol at a concentration of 1% (wt/vol). The sensitized or nonsensitized mice were challenged as described above; however, 1 h prior to 1% TNCB application 30 μl of 1% DEX, 1% CS, 1% FK-506, or solvent control was applied on the ear, and ear thickness measurement and immunolabeling were performed at 24 h and 48 h after the TNCB painting. The increase of relative Langerhans cell size and the decrease of relative Langerhans cell density were assessed in each group. Mean percentage inhibition of increased Langerhans cell size was calculated by the following formula:

$$\text{Mean percentage inhibition of increased LC size} = \frac{\text{LC size in solvent-treated group} - \text{LC size in drug-treated group}}{\text{LC size in solvent-treated group} - 100} \times 100(\%)$$

Mean percentage restoration of decreased Langerhans cell density was calculated by the following formula:

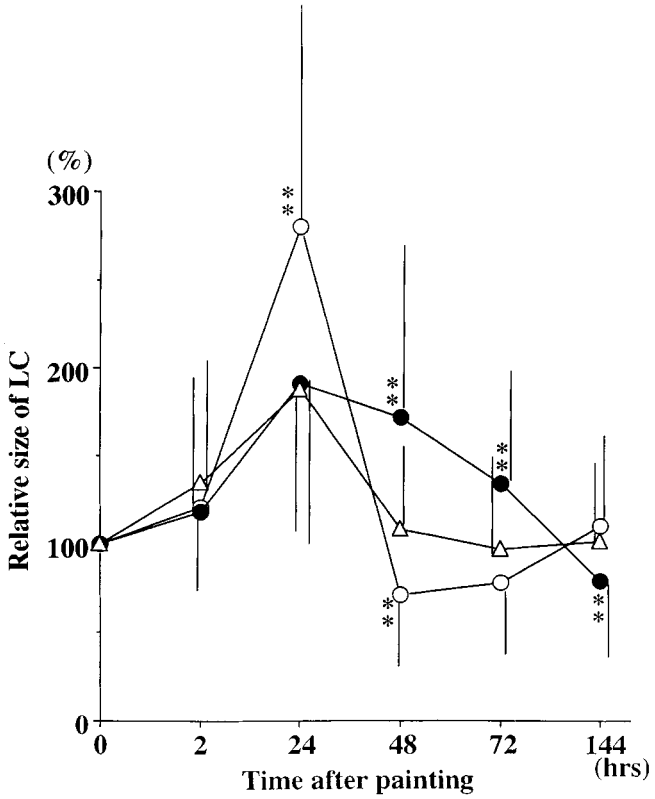

Figure 3. Time course of relative size of Langerhans cell after 1% TNCB painting in the unsensitized and sensitized mice. TNCB application induced a rapid and significant increase of the relative size of Langerhans cell at 24 h in the unsensitized mice. The enlargement of Langerhans cell size then rapidly normalized or even became smaller at 48 h. In the efferent phase the enlargement of Langerhans cell size was significantly smaller, but it lasted longer than that in the afferent phase. The enlargement of Langerhans cell was detected at 24 h even after the application of olive oil only. △, Olive oil only (n = 55); ○, TNCB painting (unsensitized group) (n = 28); ●, TNCB painting (sensitized group) (n = 30). Data show mean ± SD. **p < 0.01.

$$\text{Mean percentage inhibition of increased LC density} = \frac{\text{LC density in solvent-treated group} - \text{LC density in drug-treated group}}{\text{LC density in solvent-treated group} - 100} \times 100(\%)$$

Statistical analysis Student's t test was used to analyse significance and a p value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Morphologic and numerical alteration of Langerhans cells are differentially regulated in the afferent and efferent phase of CHS We first examined the ear thickness increments after 1% TNCB painting in the unsensitized (afferent phase) and sensitized (efferent phase) mice. In sensitized mice, marked ear swelling was observed 24 h after the TNCB application at a peak of 48 h (**Fig 1**). In unsensitized mice the application of 1% TNCB induced slight but significant swelling at 24 h after painting when compared with control olive oil-painted mice (**Fig 1**). In accordance with the ear swelling, the epidermal density of Ia^+ Langerhans cells was significantly reduced at 24–72 h in the sensitized mice (**Fig 2**). The number of Langerhans cells also tended to decrease in the unsensitized mice; however, the reduction was transient and was not statistically significant (**Fig 2**). With regard to the morphologic alteration of Langerhans cells, the TNCB application resulted in a rapid and significant increase of the relative size of Langerhans cells at 24 h in the unsensitized mice (**Fig 3**). The enlargement of Langerhans cell size then rapidly normalized or even became smaller at 48 h. In contrast, TNCB application also

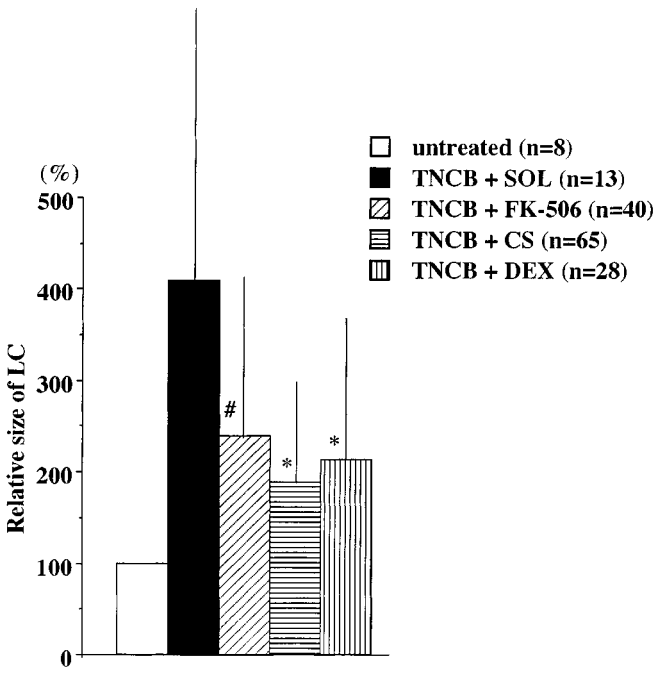

Figure 4. Effects of topical FK-506, CS, and DEX on the enlargement of Langerhans cell size in the unsensized mice. The solvent control did not affect the activation pattern of Langerhans cells, and the relative size of Langerhans cells increased in the unsensitized mice. FK-506, CS, and DEX each clearly inhibited the enlargement of Langerhans cell size induced by the TNCB application. Data show mean ± SD. *p < 0.05; #p = 0.0702.

increased the relative size of Langerhans cells at 24 h in the sensitized mice, but the increase was much less than that of the unsensitized mice (**Fig 3**). The increase of relative size of Langerhans cells at 24 h in the sensitized mice was not statistically significant compared with the control olive oil application group, because the enlargement of Langerhans cells was detected at 24 h even after the application of olive oil only (**Fig 3**). In the sensitized mice, however, the enlargement of Langerhans cells lasted longer up to 72 h, and thus significant increments of Langerhans cell size were recognized at 48 h and 72 h when compared with olive oil application (**Fig 3**).

The critical difference between the afferent and efferent phases of CHS was the absence or presence of dermal infitrates, which were mainly composed of lymphoid cells, neutrophils, and eosinophils. In the afferent phase TNCB application induced rapid and remarkable enlargement of Langerhans cell size but not marked reduction of Langerhans cell density. In the efferent phase the enlargement of Langerhans cell size was significantly smaller, but it lasted longer than that in the afferent phase. In addition, there was a marked reduction of Langerhans cell density in the efferent phase. The difference of activation pattern may be due to the direct effects of infiltrated leukocytes on the Langerhans cells in the efferent phase. Alternatively, in the efferent phase, the fully activated Langerhans cells may smoothly and rapidly emigrate from the epidermis as early as 24 h, and it is possible that residual Langerhans cells are an incompletely activated or incompletely enlarged population at 24 h after the TNCB application.

Inhibition of Langerhans cell activation by topical application of immunosuppressive drugs Using this assay system, we next examined the effects of FK-506, CS, and DEX on the activation patterns of Langerhans cells in the afferent and efferent phases of CHS. The immunosuppressive activity of these agents was very potent because topical application of either 1% FK-506, 1% CS, or 1% DEX prior to the TNCB challenge in the sensitized mice almost completely inhibited the ear swelling compared with solvent control treatment (data not shown; Furue and Katz, 1989; Furue

Table I. Effects of topical FK-506, CS, and DEX on the Langerhans cell activation in the unsensitized (afferent phase) and sensitized (efferent phase) mice

	Mean percentage inhibition of increased Langerhans cell size		Mean percentage restoration of decreased Langerhans cell density	
	24 h	48 h	24 h	48 h
Unsensitized mice (afferent phase)				
FK-506	54.9%	–0.1%	76.6%	100%
CS	71.2%	40.6%	69.0%	68.6%
DEX	63.5%	125.6%	–0.1%	–221.9%
Sensitized mice (efferent phase)				
FK-506	37.9%	96.8%	ND	95.3%
CS	46.8%	98.1%	ND	52.2%
DEX	7.4%	77.1%	ND	–23.3%

et al, 1994). We assayed the effects of drugs at 24 h and 48 h after the TNCB application. The solvent control did not affect the activation pattern of Langerhans cells induced by the TNCB painting, and the relative size of Langerhans cells increased in association with reduction of density in both the unsensitized (**Fig 4**) and the sensitized (data not shown) mice. Very interestingly, all the drugs clearly inhibited the enlargement of Langerhans cell size induced by the TNCB application in both the unsensitized and the sensitized mice (**Fig 4**, **Table I**). In keeping with the results of time course experiments of Langerhans cell size (**Fig 3**), the inhibitory effects on Langerhans cell size by drugs were stronger at 24 h than at 48 h in the unsensitized mice (**Table I**). In the sensitized mice the inhibitory effects were more clearly recognized at 48 h than at 24 h (**Table I**). With regard to the decreased Langerhans cell density, FK-506 and CS were capable of restoring the reduction of Langerhans cell density; however, DEX not only could not restore the decreased Langerhans cell density, but further induced its reduction (**Table I**).

FK-506, CS, and DEX are potent immunosuppressive drugs that are very useful therapeutic modalities in the dermatologic field. CS has been shown to directly downregulate the accessory and antigen-presenting cell function of Langerhans cells without affecting the viability (Furue and Katz, 1988a, b). DEX also directly decreases the number of Langerhans cells *in vivo* and markedly causes the viability of Langerhans cells to deteriorate *in vitro* (Furue and Katz, 1989). The cytotoxic (perhaps apoptosis-inducing) effects on Langerhans cells by DEX may explain the continuous reduction of Langerhans cell density after topical application (**Table I**). As all the drugs inhibited the enlargement of Langerhans cell size induced by TNCB stimulation not only in the afferent phase but also in the efferent phase, the present data suggest that immunosuppressive drugs are likely to directly affect the activation of Langerhans cells, and that the inhibitory effects on Langerhans cell activation may partly participate in the profound immunosuppressive activity of the agents.

Previous studies have disclosed that the survival and functional maturation of Langerhans cells are profoundly supported by keratinocyte-derived cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1, and tumor necrosis factor-α (TNF-α). GM-CSF enhances the survival and APC function of Langerhans cells (Witmer-Pack *et al*, 1987). IL-1 further augments the GM-CSF-induced functional maturation of Langerhans cells (Heufler *et al*, 1988). In contrast, TNF-α is active in maintaining the survival of Langerhans cell as potently as GM-CSF without increasing their APC function (Koch *et al*, 1990). Therefore it is possible that the immunosuppressive drugs may inhibit the release of keratinocyte-derived cytokines, which in turn suppress the early Langerhans cell activation.

In conclusion, these results indicate that the activation pattern of Langerhans cells is influenced by whether the host has been previously sensitized or not. Furthermore, the Langerhans cell activation is clearly downregulated by the immunosuppressive drugs, suggesting that the measurement of Langerhans cell activation may be one of the useful screening methods to evaluate the immnosuppuressive activity of newly developed therapeutic compounds.

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