Korean J. Toxicol.
Vol. 7, No. 2, pp. 129-139, 1991



# PREVENTIVE EFFECTS OF RED GINSENG SAPONIN ON HYPERKERATOSIS: ULTRASTRUCTURAL OBSERVATION AND LIPID ANALYSIS

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*(Received June 13, 1991)*
*(Accepted August 20, 1991)*

**ABSTRACT:** *Preventive effect of red ginseng saponin on experimentally-induced hyperkeratosis was investigated by ultrastructural observation, skin weight and epidermal lipid analysis. Hexadecane increased skin weight per unit area and epidermal lipids, free fatty acids, cholesterol and triglyceride in guinea pig skin. Topical application of ginseng saponin reduced these hyperkeratotic responses regardless of the concentration and the purity of ginseng saponin. Ultrastructurally, lipids and empty space-containing multiple horny cells were piled and nuclear remnants, desmosome, desmosomal bodies, tight junction were shown in the stratum corneum of hexadecane-treated skin. In hexadecane-treated stratum granulosum, keratohyaline granules, disintegrated desmosome and membrane-coating granules were lesser than in control or vehicle skin. Ginseng saponin application resulted in no tight junction, relatively low lipids and low spaces in horny cells and appearance of membrane-coating granules in the stratum granulosum. Our previous thought of ginseng saponin for controlling epidermal cellular proliferation in the stratum basale supports the present results, preventive effect of ginseng saponin for hyperkeratotic ultrastructural changes in the stratum corneum and in the stratum granulosum in epidermis of guinea pig skin.*
**Key words:** *Red ginseng saponin, Hyperkeratosis, Ultrastructure, Epidermis*

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# INTRODUCTION

Stratified squamous epithelium is distributed widely throughout the body and found in most locations where friction commonly encountered. The surface of the body is covered by such an epithelium, but it is different from all others of the body in that it is keratinized (Junqueria and Carneiro, 1983). Keratinization is the process whereby the living cells undergoing mitosis in the stratum basale and one of the daughter cells become converted to the dead cells of the stratum corneum. These dried cells filled with hydrophobic proteins limit water loss from the body and limit the entry of external noxious substances and microorganisms into the body (Johnson, 1984). This dried outermost layer of stratum corneum filled with fibrillar protein and thickened plasma membranes after keratinization is also called as horny layer (Becker *et al.*, 1986). Hyperkeratosis or hyperkeratinization is an excessive thickening of the horny layer of the epidermis caused by endogenous or exogenous substances (Junqueira and Carneiro, 1983).

Topical application of various noncarcinogenic hydrocarbons and mineral oils leads to hyperplasia and hyperkeratinization to mammalian skin (Hoekstra and Phillips, 1963; Brooks and Baumann, 1956). Of the saturated, straight-chained hydrocarbons, hexadecane induces the most marked changes in the skin of guinea pigs and provides a model for studying keratinization process since the response of the epidermis is localized, dramatic and predictable (Kirk and Hoekstra, 1964). In our previous papers(Kim(Jun) *et al.*, 1989, 1990), 2% total saponin solution from Korean red ginseng showed preventive effect on hexadecane-induced hyperkeratosis by controlling the enzyme activities involved in epidermal cellular metabolism, resulting in reduced amounts of abnormally accumulated lipid in the epidermis. Among red ginseng components (water extract, lipid fraction, nonlipid fraction, saponin), only saponin had beneficial effect on experimentally-induced hyperkeratosis, which was similar to the effect of oral administration of 13-cis-retinoic acid. Present study was designed to investigate whether the concentration and the purity of saponin affect its preventive effect on hyperkeratosis and how saponin changes the ultrastructure of stratum corneum and stratum granulosum of epidermis in guinea pig skin.

# MATERIALS AND METHODS

### Materials

n-Hexadecane, glutaraldehyde, sodium cacodylate, osmium tetraoxide, uranyl acetate and lead citrate were obtained from Sigma Chemical Co. (St. Louis, Mo). Triglyceride and cholesterol test kit from Iatron Laboratories, Inc. (Tokyo, Japan) and nonesterified fatty acid test kit from Nissui Pharmaceutical Co. (Tokyo, Japan) were used. All other reagents were of guaranteed reagent grade commercially available.

### Ginseng Saponin Preparation

Total ginseng saponin was prepared from water-saturated butanol extract of powdered Korean red ginseng, followed by repeated filtration after addition of

activated charcoal and methanol to obtain a pure white powder (Sanada *et al.*, 1974). Panaxadiol and panaxatriol were purified from total saponin by column chromatography and combined to be the ratio of panaxadiol(D) and panaxatriol(T) as 1.67:1 since the ratio of D/T in Korean red ginseng ranges from 1.40 to 2.75 (Kim *et al.*, 1986).

**Animal Treatment**

Female Hartley guinea pigs, 450-500g from Sam Yuk Animal Breeding Lab. (Osan, Korea), were kept under conventional laboratory conditions with commercial laboratory chow (Jeil Animal Food Ind.), tap water and fresh vegetables ad libitum and used after 10 day of acclimation. Dorsal hairs, over an area of approximately 8 $cm^2$, were shaved with electric clipper 1 day prior to the experiment. 0.5 m*l* of ginseng saponin (0.5%, 2%) and a mixture of panaxadiol and panaxatriol(2%) were topically applied to dorsal skin of each guinea pig 1 hr before the application of n-hexadecane (2 m*l*/kg B.W.) every day for 10 days. n-Hexadecane was administered every other day during the experimental period. 0.5 m*l* of 50% ethanol solution was topically applied daily as a vehicle. Animals were housed under subdued light and were killed by cervical dislocation at the 10th day of experiment. For electron microscopic observation, 2% total saponin solution was chosen for application and control animals which were not treated at all were used in addition to vehicle group and hexadecane group.

**Skin Weight Determination**

Two pieces(1.5 $cm^2$) of treated skin area from each guinea pig were excised and weighed before and after removing subcutaneous fat and muscle with a sharp scapel blade (No. 21, Keisei Medical Industrial Co., Tokyo, Japan) at the end of experiment.

**Epidermal Lipid Analysis**

Epidermis was separated from two pieces (1.5 $cm^2$) of skin for each animal by brief heat treatment (Conner and Lowe, 1983). Epidermis was minced with scissors ($<1$ $mm^3$), homogenized in the mixture of chloroform and methanol (2:1, v/v) filtered with glass fiber filter and dried under $N_2$ gas. Total lipid was measured by weighing method (Christie, 1982). Dried extract was redissolved in 1 m*l* of methanol and used for the analysis of triglyceride, cholesterol and free fatty acids. These individual lipids were determined by enzymatic methods using commercial test kits.

**Transmission Electron Microscopic (TEM) Observation**

Biopsy materials were placed in a glutaraldehyde fixative (2% glutaraldehyde in 0.1 M sodium cacodylate.buffer, pH 7.4) overnight at 4℃, rinsed in the buffer and postfixed in 1% osmium tetraoxide in the same buffer for 2 hr at 4℃. Following dehydration through a series of graded acetone, the tissues were embedded in Epon 812 (Electron Microscopy Science, Fort Washington, PA). Thin sections were cut with an ultramicrotome (Sorval MT 2-B, Ivan Sorvall Inc., Norwalk, CT), stained with uranyl acetate for 30 min and lead citrate for 15 min, and examined in an

electron microscope (Model H-500, Hitachi, Ltd., Tokyo, Japan).

## RESULTS AND DISCUSSION

### Effect of Ginseng Saponin on Skin Weight and Epidermal Lipid Content

Increase in epidermal weight per unit area and a loss in water content of epidermis were hyperkeratotic responses in guinea pig skin according to the study by Kirk and Hoekstra (1964). They reported that decreased water content was due, at least in part, to a decreased water binding potential of the water-soluble, dialyzable fraction of the cornified layer. Therefore, we measured the weight of treated area of skin before and after removing subcutaneous fat and muscle as one of the parameters for hyperkeratosis. As shown in Table 1. five time appliation of hexadecane at 2 m*l* per kg body weight increased the weight of whole skin and the weight of epidermis and dermis to 150% and 165% of vehicle value, respectively. Topical application of total ginseng saponin (0.5%, 2%) and a mixture of panaxadiol and panaxatriol significantly reduced the weight of skin ranged from 82% to 91% of hexadecane treated group ($P<0.05$).

**Table 1.** Fresh weight of guinea pig skin

| Treatment | Whole Skin | Epidermis + Dermis |
|---|---|---|
| Vehicle | 0.4561±0.034 | 0.3224±0.021 |
| HD | 0.6992±0.061 | 0.5332±0.042 |
| HD + TS, 0.5% | 0.6165±0.076[a] | 0.4521±0.069* |
| HD + TS, 2% | 0.6100±0.060* | 0.4359±0.055** |
| HD + D/T, 2% | 0.6320±0.065* | 0.4852±0.046* |

Values represent mean±SD of 6 animal skins and expressed as $g/1.5\ cm^2$. Asterisks indicate values significantly different from the value of HD (Hexadecane) group by Student's t-test; *; $P<0.05$, **; $P<0.005$.TS, Total Saponin, D/T, a mixture of Panaxadiol/Panaxatriol (1.67:1).

Increasing number of acquired and genetic disorders of keratinization appear to be accompanied by lipid abnormalities and skin scaling abnormalities seem to be related to aberrant skin lipid metabolism (Elias, 1981). Ichthyosis is a scaling dermatosis associated with abnormal lipid metabolism and there are two models presented for screening of new drugs: (a) topical application of hairless mice of lipids (cholesterol sulfate, n-alkanes) which are known to accumulate in the stratum corneum in human ichthyotic skin (Elias *et al.*, 1985) and (b) n-hexadecane - induced hyperkeratosis with epidermal proliferation in guinea pig skin (Kirk and Hoekstra, 1964).

In our previous papers (Kim(Jun) *et al.*, 1989, 1990), significant increase in epidermal lipid was found: the order of increment was free fatty acid, cholesterol and then triglyceride. Accumulation of lipid in epidermis was thought to be caused by highly proliferative epidermal cells which are stimulated by hexadecane application. By the review paper of epidermal lipids (Elias, 1981), the composition

**Table 2.** Effect of ginseng saponin on epidermal lipid in guinea pig skin

| Treatment | Total Lipid | Cholesterol | Triglyceride | Free Fatty acid |
|---|---|---|---|---|
| Vehicle | 7.23±0.29 | 0.155±0.026 | 0.373±0.075 | 0.146±0.018 |
| HD | 10.80±2.36 | 0.382±0.032 | 0.475±0.080 | 0.848±0.103 |
| HD+TS. 0.5% | 7.83±1.21* | 0.243±0.029*** | 0.381±0.065* | 0.352±0.152** |
| HD+TS. 2% | 7.51±0.37** | 0.241±0.030*** | 0.384±0.059* | 0.302±0.104*** |
| HD+D/T. 2% | 8.09±1.65* | 0.313±0.042*** | 0.386±0.041*** | 0.378±0.162** |

Values are mean±SD of 6 animals and epressed as mg/2×1.5 $cm^2$ for total lipid, cholesterol and triglyceride and μEq/2×1.5 $cm^2$ for free fatty acid. Asterisks indicate values significantly different from the value of HD (Hexadecane) group by Student's t-test; *: $P<0.05$, **: $P<0.001$, ***: $P<0.005$.

of lipids isolated from rodent, pig and human stratum corneum is surprisingly similar: phospholipids are scanty and larger quantities of sphingolipids, free sterols, sterol esters and free fatty acids are contained in stratum corneum than in stratum granulosum. Polar lipids are larger in stratum granulosum (Gray and Yardly, 1975). In present study, large increment in free fatty acid, cholesterol and triglyceride were observed by hexadecane application, which were 580%, 250% and 130% of vehicle value while total lipid was 150% of vehicle value (Table 2). Even though phospholipids, glycosphingolipids and ceramides were not determined, hexadecane-induced increment in neutral lipids indicated increase in the stratum corneum in epidermis. Reduction in epidermal lipids by ginseng saponin application was ordered as follows: free fatty acid, cholesterol and triglyceride were 36-45%, 63-82% and 80-81% of vehicle value, respectively. Total lipid in ginseng saponin-treated epidermis was similar to the value of vehicle group, which ranged from 69% to 75% of hexadecane value. From these results, it is thought that neither the concentration (0.5%, 2%) nor the purity (a mixture of panaxadiol and panaxatriol) of ginseng saponin changes the preventive effect of ginseng saponin on experimentally-induced hyperkeratosis when determined by skin weight and epidermal lipid contents.

## Effect of Ginseng Saponin on the Ultrastructure of Epidermis

All micrographs were taken from the treated area of back skin of guinea pig at 10th day of experiment. The epidermis is divided into four layers: stratum basale, stratum spinosum, stratum granulosum and stratum corneum. In our previous study (Kim(Jun) *et al.*, 1990), hexadecane induced hyperkeratinization by increasing proliferative cells in the stratum basale and the stratum spinosum. Topical application of red ginseng saponin (2% solution) reduced hyperplasia of epidermis and the numbers of horny cell layers of the stratum corneum. Since the stratum basale and the stratum spinosum did not seem to be structurally changed during keratinization except for increase in the numbers as well as the sizes of epidermal cells (Kim(Jun) *et al.*, 1990), differences in the upper layer of the stratum granulosum and the stratum corneum were observed in the present study. In control animals, the stratum corneum contained flattened nonnucleated keratinized cells (horny cell, HC) whose cytoplasm is filled with a birefringent filamentous scleroprotein, keratin. This protein present filaments embedded in a

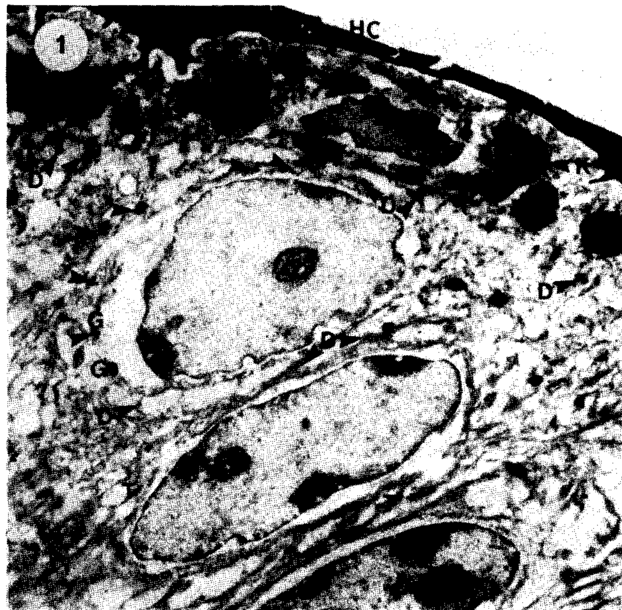


**Photo. 1.** Electron micrograph of control skin. The stratum corneum contained flattened nonnucleated keratinized cells (horny cells, HC) embedded in a dense amorphous matrix. Below horny cells, transitional cells had large keratohyaline granules (K) and nuclear remnants. The stratum granulosum contained centrally located nuclei and cytoplasm filled with keratohyaline granules, tonofibrils (T), desmosome (D) and membrane-coating granules (G). (×18,000)

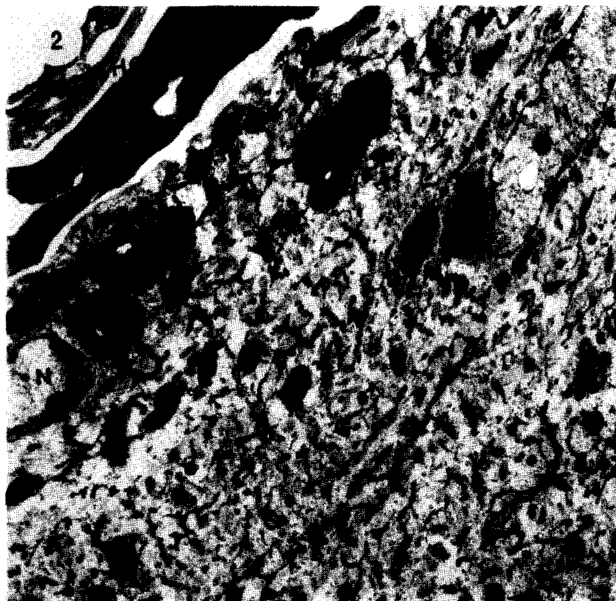


**Photo. 2.** The ultrastructure of vehicle skin showed similar to that of control animal, but well-developed tonofibrils (T) appeared. (×18,000)

dense amorphous matrix (Photo 1). Below horny cells, transitional cells which had large keratohyaline granules (K) and nuclear remnant are shown. As keratinization proceeds, the cytoplasm of the keratinocyte contains increasing numbers of autophagosomes in which cellular organelles are digested by lysosomal enzymes. This explains the loss of cell structure and the keratohyaline appearance of keratinized cells (Junqueira and Carneiro, 1983). The next lower layer, the stratum granulosum, contained centrally located nuclei and cytoplasm filled with coarse basophilic granules, keratohyaline granule (K) and membrane-coating granule (G), ovoid or rodlike appearance. Membrane-coating granules, formed in association with the Golgi apparatus, move near the upper part of the cell near its plasma membrane. They fuse with the membrane and discharge their contents into the intercellular spaces of the granular layer. Desmosomes (D) that punctuate the cell surface and tonofilament bundles (T) that play an important role in maintaining cohesion among cell were shown in the epidermis of control animals. The ultrastructure of 50% ethanol-treated skin showed similar to that of control animal, but well-developed tonofibrils appeared (Photo 2). In hexadecane-treated skin, multiple horny cells were piled with narrow intercellular spaces (IS). Lipids(L) were largely retained and there were many empty spaces in horny cells. Nuclear remnants(NR) , granules, desmosomes(arrows) and tight junctions(TJ) between the cells were shown (Photo 3a). In the stratum granulosum, keratohyaline granules (K) and disintegrated desmosomes (D) were lesser than in control or vehicle group (Photo 3b). The stratum corneum was loosely attached to each other by desmosome (D) and desmosomal body, fusiform electron dense bodies (DB,

**Photo. 3a.** The stratum corneum of hexadecane-treated skin. Multiple horny cells were piled with narrow intercellular spaces (IS). Largely retained lipids (L), empty spaces, nuclear remnants (NR), granules and tight junction (TJ) between the cells were shown. (×15,000)

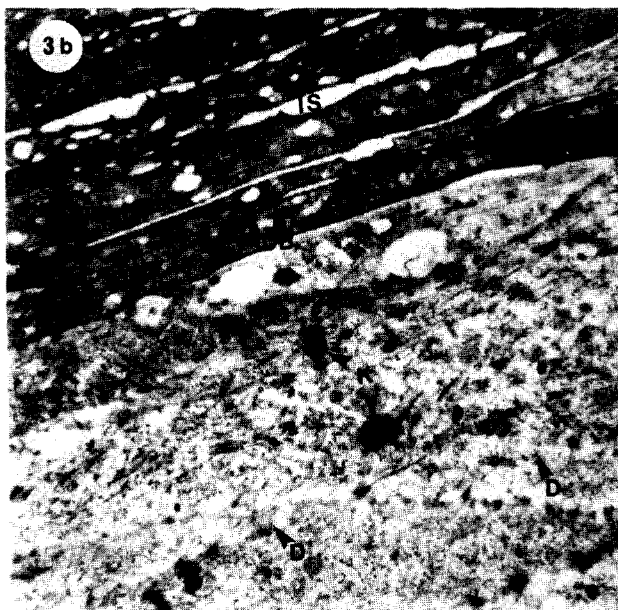


**Photo. 3b.** The stratum corneum and the stratum granulosum of hexadecane-treated skin. Horny cells were loosely attached each other by desmosome (D) and desmosomal bodies (DB, arrows). In the stratum granulosum, keratohyaline granules (K) and disintegrated dosmosomes (D) were lesser than in control or vehicle animals. (×15,000)

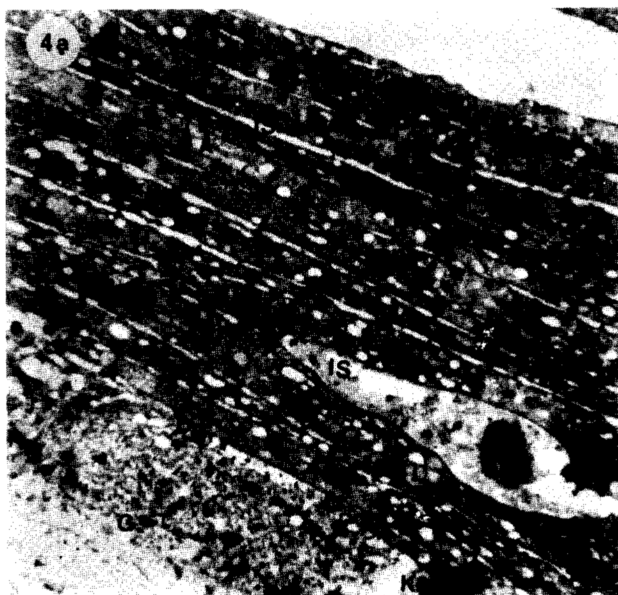


**Photo. 4a.** The stratum corneum of ginseng saponin-treated skin. Partially digested cellular organelles in intercellular spaces (IS), desmosomal bodies (DB) between the cells, keratohyaline granules (K) and lipids (L) appeared. No tight junction and relatively low lipid and low empty spaces were observed as compared with that of hexadecane-treated skin. (×15,000)

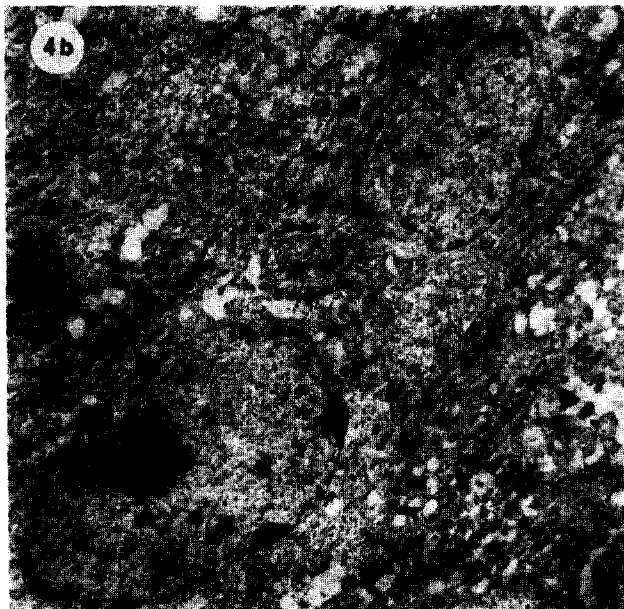


**Photo. 4b.** The stratum granulosum of ginseng saponin-treated skin. Tonofibrils (T), desmosome (D) and membrane-coating granules (G) along the cell surface were shown. (×14,000)

arrows), which was different from hyperkeratosis induced by oleic acid. Oleic acid-induced comedones occurred in two different ways initially, by desmosomes and desmosomal bodies, which loosely held the horny cells together; later by tight junctions, which tightly bound the horny cell together (Woo-Sam, 1977). The stratum corneum of ginseng saponin-treated skin had partially digested cellular organelles in the intercellular space (IS), desmosomal bodies (DB) between the cells, keratohyaline granules (K) and lipids (L) (Photo 4a). However, no tight junction between horny cells, relatively low lipids retained and low empty spaces appeared. As shown in Photo 4b, there were tonofibrils (T) and desmosomes (D) and membrane-coating granules (G) along the cell surface. Membrane-coating granules containing hydrolytic enzymes are normally secreted between the keratinocytes and create an intercellular space, which separates the cells and prepares them for desquamation (Lazarus *et al.*, 1975). Presumably, disappearance of membrane-coating granules and appearance of nuclear remnants in hexadecane-treated skin and appearance of membrane-coating granules in saponin-treated epidermis explain that keratinocytes could not be hydrolyzed completely in the stratum granulosum and partially digested cellular organelles and nuclear remnants appeared in the stratum corneum by hexadecane treatment. Ginseng saponin prevented these ultrastructural changes caused by hexadecane-stimulated epidermal cellular proliferation. Our results support two recent dermatologic studies about reduction in both the sizes and the numbers of oleic acid-induced comedones in ginseng-treated rabbit ears (Im *et al.*, 1990; Sung *et al.*, 1990).

## ACKNOWLEDGEMENTS

We wish to thank the Staffs in Electron Microscopy Laboratory, School of Medicine, Yonsei University for their helps in electron microscopic observation and Professor J. K. Park, Department of Dermatology, School of Medicine, Chung Nam National University for the valuable discussion.

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