Increased Proliferative Response of Peripheral Blood Mononuclear Cells and T Cells to *Streptococcus mutans* and Glucosyltransferase D Antigens in the Exacerbation Stage of Recurrent Aphthous Ulcerations

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Background and Purpose: Cytotoxic T lymphocyte-induced lysis of virus-infected oral epithelial cells has been shown to be a cause of early ulcer formation in recurrent aphthous ulcerations (RAU). To test whether bacteria and their associated antigens are involved in the disease process of RAU, the proliferative response (PR) to different streptococcal species in peripheral blood mononuclear cells (PBMC) and T cells isolated from RAU patients at the exacerbation stage was determined.

Methods: PBMC and T cells were isolated from 39 patients with RAU, 21 patients with erosive oral lichen planus (EOLP, disease control group), and 22 healthy subjects (normal control group). *Streptococcus mutans, Streptococcus sanguis, Streptococcus oralis, Streptococcus gordonii, Streptococcus mitis* and their associated antigen, glucosyltransferase D (GtfD), were used to stimulate isolated PBMC and T cells in *in vitro* proliferation assays.

Results: PBMC and T cells isolated from RAU patients at the exacerbation stage showed a significantly higher PR to *S. mutans* antigen and GtfD than those isolated from EOLP patients or healthy control subjects ($p < 0.05$). GtfD was a more potent stimulation antigen than *S. mutans*. However, elevated PRs to *S. mutans* antigen and GtfD were transient and present only in the exacerbation stage of RAU. These elevated PRs declined to normal levels in the postexacerbation and convalescence stages of RAU. Furthermore, the GtfD-stimulated PR in PBMC and T cells was correlated with the severity of RAU.

Conclusion: In addition to viral infections, streptococci and their associated antigen, GtfD, may be involved in the disease process of RAU, especially in the exacerbation stage.

Recurrent aphthous ulcerations (RAU) are common in the oral mucosa and are characterized by recurrent and painful ulcerations of the oral nonkeratinized mucosa. The etiology of RAU is still obscure; however, alterations of humoral and cellular immunities in patients with RAU have been reported [1–4].

It has long been believed that RAU have a viral or bacterial etiology. Although a number of different viruses have been implicated in RAU, the four herpesviruses — herpes simplex virus (HSV), varicella zoster virus (VZV), human cytomegalovirus (HCMV), and Epstein-Barr virus (EBV) — are most frequently associated. Studies have shown significantly higher serum anti-HSV, anti-VZV and anti-HCMV antibody levels in RAU patients than in normal controls [5–9]. Furthermore, HSV, VZV, HCMV and EBV DNAs have been detected by polymerase chain

reaction (PCR) of biopsies of ulcerative and preulcerative oral aphthae from RAU patients [6, 9–11]. We have recently also demonstrated the presence of EBV-DNA and EBV-associated antigens in preulcerative oral aphthous tissues using *in situ* hybridization (ISH) and immunohistochemistry [11]. The presence of herpesvirus DNAs and their associated antigens in oral aphthous tissues suggests that herpesviruses may be the etiologic agents of RAU.

Some researchers have investigated the association of bacteria with RAU, but the results are controversial. Several studies found raised levels of antibody against certain viridans streptococcal strains in RAU patients compared with control subjects [12–16]. Conversely, subsequent studies found approximately equal or significantly lower levels of antibodies against various streptococcal strains in RAU patients compared with normal controls [17–19]. Barile et al first hypothesized an association between RAU and hypersensitivity to putative strains of *Streptococcus sanguis* [20]. Bacteria identified as *S. sanguis* are isolated, frequently in pure culture, from aphthous lesions, and delayed-type hypersensitivity skin reactions occur in RAU patients after intradermal injection of vaccines prepared from these isolates [20]. Lymphocytes from RAU patients, but not normal controls, have been shown to induce increased leukocyte migration inhibition in response to streptococcal and oral mucosal antigens that correlates with exacerbation of the RAU [15, 16]. However, this observation was not confirmed by Gadol et al [21]. In addition, lymphocytes from RAU patients show a decreased or equal proliferative response (PR) to streptococcal antigens [22–24]. These discrepant results indicate a need for further study of the role of streptococcal antigens in the disease process of RAU.

Our previous studies showed that herpesviruses such as HCMV and EBV, and cytotoxic T lymphocytes (CTL) may be involved in the early stage of RAU [9, 11]. CTL-induced lysis of virus-infected oral epithelial cells may be the cause of early ulcer formation in RAU. Oral bacteria may secondarily infect the early ulcerative oral aphthous lesion through the mucosal break. If bacteria and their associated antigens are involved in the disease process of RAU, immunocompetent cells isolated from RAU patients at the exacerbation stage should show a specific response to these bacteria and their associated antigens. Therefore, in this study we tested whether streptococci and their associated antigens could stimulate a specific PR in peripheral blood mononuclear cells (PBMC) and T cells isolated from RAU patients at the exacerbation stage. PBMC and T cells were isolated from patients with RAU or erosive oral lichen planus (EOLP) and normal control subjects. Because different species of streptococci have been found in the oral cavity, including the mucosal surface,

saliva and dental plaque [25], we first examined the PR of PBMC to five different streptococcal stimulation antigens. Because the results of tests of association between *S. sanguis* and RAU, including lymphoproliferative response, leukocyte migration inhibition and serum antibody response to *S. sanguis* have already been reported [18–24], while the PRs of PBMC and T cells from RAU patients to *Streptococcus mutans* (GS-5) and its associated glucosyltransferase D (GtfD) antigens have not, we further examined whether *S. mutans* and GtfD could also stimulate a higher PR in PBMC and T cells from RAU patients than in EOLP patients or healthy control subjects.

Materials and Methods

Subjects

Thirty-nine RAU patients (16 men and 23 women; mean age 31 ± 10 yr; range, 16–61 yr), 21 EOLP patients (9 men and 12 women; mean age 36 ± 9 yr; range, 21–65 yr), and 22 normal control subjects (10 men and 12 women; mean age 21 ± 2 yr; range, 20–22 yr) were included in this study. All RAU patients had had at least one episode of oral ulcerations per month during the year preceding enrollment. The development of RAU is divided into three stages: exacerbation (10 patients), from the onset of oral mucosal ulceration to the day of maximum ulcer pain; postexacerbation (22 patients), from the day of maximum ulcer pain to the complete healing of the oral ulceration; and convalescence (8 patients), 1 or 2 weeks after remission of the oral mucosal ulceration. The severity of RAU was subdivided according to the criteria of Lehner [26] into major aphthous ulcerations, defined as recurrent painful oral ulcerations with a diameter of more than 1 cm (12 patients), and minor aphthous ulcerations, defined as recurrent painful oral ulcerations less than 1 cm in diameter (27 patients). Twenty-one EOLP patients were selected according to the following criteria: a typical clinical presentation of radiating grayish-white striae of Wickham and erosion or ulceration on the oral mucosa, and biopsy specimens characteristic of OLP, that is, hyperkeratosis or parakeratosis, a slightly acanthotic epithelium with liquefaction degeneration of basal epithelial cells, and a pronounced bandlike lymphocytic infiltrate in the lamina propria. The normal control group included 22 healthy dental students without any manifestations of oral mucosal disease. All patients were diagnosed and treated in the Department of Oral Pathology and Oral Diagnosis of National Taiwan University Hospital. None of them were taking any medication at the time of enrollment.

Purification of recombinant GtfD

Recombinant GtfD expressed in *Escherichia coli* was purified by chromatography on a Ni^{2+} affinity resin. The plasmid, pYND72-His, expressing *gtfD* with a 7-His tag immediately C-terminal to the putative signal sequence (amino acids 1–29) was constructed as described in a recent report [27]. The bacteria were grown to an A_{550} of 0.4 to 0.5, and induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 2.0 mM. The cultures were grown for an additional 4 hours and then harvested. Cell pellets were resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9) and a cell lysate was prepared by disrupting the cells by sonication. The cell debris was removed by centrifugation at $15,000g$ for 30 minutes. A small disposable column was packed with 2.5 mL of chelating Sepharose Fast Flow resin (Pharmacia Biotech Inc., Piscataway, NJ, USA) and charged with 50 mM $NiSO_4$, according to the pET instruction manual provided by the manufacturer. Further steps in the purification of His-GtfD were performed according to the pET instruction manual. The His-GtfD protein was finally eluted with elution buffer (0.5 M imidazole, 0.4 M NaCl, 20 mM Tris-HCl pH 7.9), dialyzed against 100 volumes of phosphate-buffered saline (PBS) with three changes, and stored at 4°C. Homogeneity of the purified protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining or activity staining using periodic acid-Schiff reagent [28]. The bands were analyzed with an Electrophoresis Documentation and Analysis System 120 (Scientific Imaging Systems, Eastman Kodak Co., Rochester, NY, USA). Protein concentrations were determined using a modified method of Lowry et al [29], with bicinchoninic acid as the colorimetric detection reagent (BCA Protein Assay Reagent, Pierce, Rockford, IL, USA).

Stimulation antigens

S. mutans (GS-5), *S. sanguis* (ATCC 10556), *Streptococcus oralis* (ATCC 35037), *Streptococcus gordonii* (ATCC 12396) and *Streptococcus mitis* (ATCC 903) were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA). All stimulation antigens used for proliferation assays, including GtfD and reagents, exhibited undetectable endotoxin levels (< 30 pg/mL) as determined by the Limulus amebocyte lysate assay (Sigma, St. Louis, MO, USA).

Cell preparation and proliferation assay

Peripheral blood samples were collected from patients and normal control subjects. PBMC were isolated by Ficoll-Hypaque centrifugation. Suspensions (2×10^5 cells/50 μL) of PBMC in RPMI 1640 medium (Gibco BRL Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco BRL) (complete RPMI medium) were irradiated at 4,500 rads with an x-ray irradiator (Hitachi Medical Co., Tokyo, Japan) to inhibit proliferation, and used as accessory cells in T-cell proliferation assays. T cells were enriched directly from whole blood by antibody-mediated separation with RosetteSep (StemCell Technologies Inc., Vancouver, BC, Canada). The enriched T-cell fractions were collected and used in proliferation assays.

PBMC were washed and resuspended in AIM-V (Gibco BRL) supplemented with 2 mM L-glutamine, penicillin (100 μg/mL), streptomycin sulfate (100 μg/mL), and 2% serum replacement product TCH (Celox, St. Paul, MI, USA). PBMC (2×10^5 cells per well) were cultured in 96-well round-bottomed plates (Costar, Cambridge, MA, USA) in a total volume of 200 μL. Purified T cells (1×10^5 cells per well) were cultured in the presence of irradiated autologous PBMC (2×10^5 cells per well) in RPMI 1640, supplemented with 2% fetal calf serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, penicillin (100 μg/mL), streptomycin sulfate (100 μg/mL), and 2% TCH. Three replicates of each culture were incubated with *S. mutans* (1×10^6 colony forming units [CFU] or 2×10^5 CFU), *S. sanguis* (1×10^6 CFU), *S. oralis* (1×10^6 CFU), *S. gordonii* (1×10^6 CFU), *S. mitis* (1×10^6 CFU), recombinant GtfD (10 μg/mL), or unsupplemented controls.

We first examined the PR of PBMC isolated from RAU patients at the exacerbation stage to *S. mutans*, *S. sanguis*, *S. oralis*, *S. gordonii* and *S. mitis* antigens. We then examined the PR of PBMC and T cells isolated from RAU patients at different clinical stages, EOLP patients, and healthy control subjects using *S. mutans* and GtfD as the stimulation antigens. GtfD preferentially stimulates a higher PR in T cells than GtfB or GtfC. The differential response to GtfD *vs* GtfC at the cellular level is correlated with the response of salivary or serum antibody response to these Gtf molecules [27]. Therefore, only GtfD, but not GtfB or GtfC, was used as a stimulation antigen for proliferation assays in this study. Incubation was performed at 37°C in a humidified atmosphere with 5% CO_2 for 5 days. Each culture was treated with 0.2 μCi (7.4 kBq) of [^{3}H]-thymidine (Amersham International, Little Chalfont, Bucks, UK) 18 hours before harvesting. Cultures were harvested into 96-unifilter GF/C plates using a FilterMate cell harvester (Packard, Meriden, CN, USA), dried at 50°C for 30 minutes, and 30 μL of Microscint (Packard) was added per well. [^{3}H]-Thymidine incorporation was measured using a Packard microplate scintillation counter. PR was expressed as the stimulation index (mean ± standard error of the

mean), calculated as the mean counts per minute in antigen-stimulated cultures divided by counts per minute in antigen-free cultures.

Statistical analyses

The PRs of PBMC isolated from either RAU patients at the exacerbation stage or normal control subjects to different streptococcal antigens were compared between groups using the Wilcoxon signed rank test. Differences between the PRs of PBMC and T cells to either *S. mutans* or GtfD were analyzed using the Mann-Whitney rank sum test between RAU patients and healthy control subjects or EOLP patients. The result was considered significant if the *p* value was less than 0.05.

Results

In this study, we first examined the PR of PBMC isolated from four RAU patients at the exacerbation stage and four normal control subjects to five different streptococcal stimulation antigens. No significant difference in the PR of PBMC isolated from either RAU patients or normal control subjects was found with *S. oralis*, *S. gordonii* or *S. mitis* (*p* > 0.05). However, when stimulated with either *S. mutans* or *S. sanguis*, the PR in PBMC from RAU patients was significantly higher than in normal control subjects (*p* < 0.05) (Table 1).

We then examined the PRs of PBMC and T cells isolated from RAU patients at different clinical stages, EOLP patients, and normal control subjects using *S. mutans* and GtfD as the stimulation antigens. As shown in Table 2, GtfD was a more potent stimulation antigen than *S. mutans* in the proliferation assays. In general, when either *S. mutans* antigen or GtfD was used as the stimulation antigen, PBMC and T cells isolated from RAU patients at the exacerbation stage

exhibited a higher PR than those isolated from RAU patients at either the postexacerbation or convalescence stage. In addition, no significant difference in the PR of PBMC or T cells was found between RAU patients at either the postexacerbation or convalescence stage and EOLP patients or healthy controls, when either *S. mutans* antigen or GtfD was used as the stimulation antigen. *S. mutans* antigen stimulated a significantly higher PR in PBMC (*p* < 0.05) or T cells (*p* < 0.05) from RAU patients at the exacerbation stage than in those from EOLP patients or normal controls. When stimulated with GtfD, PRs of PBMC (*p* < 0.05) or T cells (*p* < 0.001) isolated from RAU patients at the exacerbation stage were also significantly greater than those from EOLP patients or normal controls (Table 2).

When RAU patients were further divided into major and minor type subgroups, only GtfD stimulated a higher PR in PBMC (*p* < 0.05) or T cells (*p* < 0.05) from major type RAU patients than in those from minor type RAU patients or normal controls. However, when *S. mutans* was used as a stimulation antigen, no significant difference in the PR of PBMC or T cells was found between major type RAU patients and minor type RAU patients or normal control subjects (Table 3).

Discussion

Four herpesviruses — HSV, VZV, HCMV and EBV — have been implicated in the pathogenesis of RAU. HSV-1 was detected by PCR in biopsies of oral aphthae from RAU patients [6]. VZV-like DNA has been detected in all oral ulcer biopsy specimens from both RAU and Behcet's disease (BD) patients [10]. Our previous studies using PCR have shown the presence of HCMV and EBV genomic DNA in preulcerative oral aphthous tissues from RAU or BD patients. HCMV and

Table 1. Proliferative responses of peripheral blood mononuclear cells (PBMC) isolated from four patients with recurrent aphthous ulcerations (RAU) at the exacerbation stage and four normal control subjects to five different streptococcal stimulation antigens

Stimulation antigens (1 x 10⁶ CFU)	Proliferative response (PBMC stimulation index) (Mean ± SEM)		
	RAU patients (n = 4)	Normal controls (n = 4)	*p* value
Streptococcus mutans	15.8 ± 3.6	4.5 ± 1.2	< 0.05
Streptococcus sanguis	15.6 ± 3.7	4.4 ± 1.3	< 0.05
Streptococcus oralis	12.4 ± 3.9	4.0 ± 1.5	NS
Streptococcus gordonii	12.9 ± 5.3	4.1 ± 1.2	NS
Streptococcus mitis	9.9 ± 3.2	3.6 ± 1.0	NS

*From the onset of oral mucosal ulceration to the day of maximal ulcer pain. CFU = colony forming unit; NS = not significant.

Table 2. Proliferative response of peripheral blood mononuclear cells (PBMC) and T cells isolated from patients with recurrent aphthous ulcerations (RAU) at different clinical stages, patients with erosive oral lichen planus (EOLP), and normal control subjects

Antigens	Proliferative response (stimulation index), mean ± SEM									
	PBMC					T cells				
	RAU			EOLP controls	Normal controls	RAU			EOLP controls	Normal controls
	Exacerbation	Postexacerbation	Convalescence			Exacerbation	Postexacerbation	Convalescence		
Streptococcus mutans (2 x 10⁵ CFU)	8.9 ± 2.8* (n = 10)	2.5 ± 0.3 (n = 9)	4.6 ± 0.9 (n = 8)	2.9 ± 0.4 (n = 11)	3.8 ± 1.1 (n = 22)	10.4 ± 3.7* (n = 8)	7.9 ± 4.2 (n = 10)	6.2 ± 1.9 (n = 8)	3.5 ± 1.0 (n = 18)	4.2 ± 1.2 (n = 22)
GtfD (10 µg/mL)	32.7 ± 11.3* (n = 8)	19.7 ± 5.8 (n = 14)	19.5 ± 9.7 (n = 8)	13.0 ± 3.7 (n = 9)	15.5 ± 2.7 (n = 22)	47.3 ± 7.1† (n = 6)	14.5 ± 3.8 (n = 6)	13.4 ± 6.6 (n = 8)	10.9 ± 2.6 (n = 16)	8.5 ± 1.0 (n = 22)

*$p < 0.05$ and †$p < 0.001$, analyzed by Mann-Whitney rank sum test. CFU = colony-forming unit.

Table 3. Proliferative response of peripheral blood mononuclear cells (PBMC) and T cells isolated from patients with major or minor type recurrent aphthous ulcerations (RAU) and normal control subjects

Antigens	Proliferative response (stimulation index), mean ± SEM							
	PBMC				T cells			
	Major type RAU	Minor type RAU	Normal controls	p value	Major type RAU	Minor type RAU	Normal controls	p value
Streptococcus mutans (2 x 10⁵ CFU)	7.9 ± 3.6 (n = 8)	4.5 ± 0.7 (n = 19)	3.8 ± 1.1 (n = 22)	NS	12.3 ± 8.4 (n = 5)	7.1 ± 1.6 (n = 21)	4.2 ± 1.2 (n = 22)	NS
GtfD (10 µg/mL)	37.1 ± 9.5 (n = 12)	13.8 ± 3.5 (n = 18)	15.5 ± 2.7 (n = 22)	< 0.05	32.6 ± 7.4 (n = 10)	14.9 ± 4.0 (n = 18)	8.5 ± 1.0 (n = 22)	< 0.05

NS = not significant; Major type RAU = oral ulcerations > 10 mm in diameter; Minor type RAU = oral ulcerations < 10 mm in diameter.

EBV DNAs were detected in five of 13 patients; two had both viral DNAs, three had only HCMV DNAs, and the other three had only EBV DNAs in the tissue specimens [9, 11]. Furthermore, EBV DNAs were detected in one of five PCR-positive cases and were identified by ISH in the nuclei of some basal and parabasal epithelial cells and lymphocytes both within the blood vessels and in the lamina propria. In the ISH-positive case, Epstein-Barr nuclear antigen (EBNA) and EBV/C_3d receptor (C_3dR) were also identified in the nuclei of some basal and parabasal epithelial cells and lymphocytes in the lamina propria and/or in the epithelium [11]. In the same study, EBV DNAs were also detected by PCR in two samples of peripheral blood lymphocytes and one sample of plasma from the five PCR-positive patients, suggesting that lymphocytes may be the reservoir for latent EBV infection and that EBV shedding may occur in the plasma [11]. Together, these results suggest that the basal and parabasal epithelial cells of preulcerative oral aphthous lesions may be infected by EBV through EBV-infected lymphocytes. Our histologic examination of preulcerative oral aphthous tissue specimens showed mononuclear lymphoid cells scattered around the vacuolated and degenerating basal and lower spinous cells. In addition, EBV particles were not found in the epithelium by electron microscopy, nor was EBV shedding detected by PCR in the saliva of EBV-DNA-positive cases [30]. These results suggest that EBV-infected epithelial cells may be attacked and lysed by CTL, and CTL-induced lysis of the EBV-infected epithelial cells, but not virus-induced cytolysis, may be the main mechanism causing early oral ulcer formation.

Many investigators have studied the role of oral viridans streptococci in the pathogenesis of RAU, either as a direct pathogen or as an antigenic stimulus culminating in the genesis of antibodies that may conceivably cross-react with keratinocyte antigenic determinants [19, 23]. The initial L-form isolate from RAU patients was typed as *S. sanguis* [20], but later analysis found that this organism was actually a strain of *S. mitis* [31]. While some studies have demonstrated elevated antibody titers to viridans streptococci in RAU patients [12–16], others have yielded contradictory results [17–19]. Furthermore, lymphocytes from RAU patients show a decrease or no difference in mitogenic response to streptococcal antigens as compared with normal controls [22–24].

This study showed that streptococci such as *S. mutans, S. sanguis, S. oralis, S. gordonii* and *S. mitis* could elicit an increased PR in PBMC from RAU patients at the exacerbation stage. *S. mutans* antigens and GtfD could also stimulate significantly higher PRs in PBMC and T cells from RAU patients at the exacerbation stage than from EOLP patients or normal control subjects. GtfD usually induced a higher PR in PBMC and T cells isolated from RAU patients at different clinical stages than *S. mutans* antigen, indicating that GtfD is a more potent mitogen than *S. mutans* antigen. However, the elevated PRs to *S. mutans* antigen and GtfD were transient and present only in the exacerbation stage of RAU; they declined to normal levels in the postexacerbation and convalescence stages of RAU. Furthermore, the GtfD-stimulated PR of PBMC and T cells was in proportion to the severity of RAU. Based on these findings, we hypothesized that viruses are involved in the preulcerative and early exacerbation stages of RAU and that bacteria are involved in the middle and late exacerbation stages of RAU. In the preulcerative stage of RAU, EBV infects the basal and parabasal oral epithelial cells through EBV-infected lymphocytes. CTL-induced lysis of EBV-infected oral epithelial cells may cause initial oral ulcer formation. After ulcer formation, bacteria such as streptococci may secondarily infect ulcerative oral aphthous lesions through mucosal breaks. Penetration of streptococci and their associated antigens into the ulcerative oral mucosa further initiates specific immune responses that produce many CTLs in both the epithelium and lamina propria of the oral mucosa. These newly generated CTLs, in turn, lyse the adjacent oral epithelial cells and cause further oral ulcer formation in the middle and late exacerbation stage of RAU. In the postexacerbation stage, the T cell PR subsides to the normal level. This gives the oral epithelial cells at the ulcer margin an opportunity to proliferate and unite the mucosal break, finally resulting in the healing of the oral ulceration. This hypothesis is also supported by our previous study, which showed a significant increase in the percentages of $CD3^+$, $CD4^+$, $CD4^+$ interleukin-2 receptor$^+$ ($CD4^+$IL-2R$^+$, activated $CD4^+$ cells), and $CD8^+$IL-2R$^+$ cells (activated $CD8^+$ cells) in peripheral blood samples of RAU patients in the exacerbation stage compared with those in normal controls. In addition, the elevated percentages of $CD3^+$, $CD4^+$IL-2R$^+$, and $CD8^+$IL-2R$^+$ cells in peripheral blood samples of RAU patients in the exacerbation stage returned to normal levels in RAU patients in the postexacerbation stage [32].

This study showed that streptococci and their associated antigens could stimulate significantly increased PRs of PBMC and T cells isolated from RAU patients at the exacerbation stage. These findings suggest that bacteria such as streptococci and their associated antigens may be involved in the disease process of RAU, especially in the middle and late exacerbation stages.

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