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Comparison of Neutral Proteases and Collagenase Class I as Essential Enzymes for Human Islet Isolation

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Background. Efficient islet isolation requires synergistic interaction between collagenase class I (CI) and class II (CII). The CI degradation alters the ratio between CI and CII and is responsible for batch-to-batch variations. This study compares the role of neutral protease (NP) plus clostripain (CP) with CI as essential enzymes for human islet isolation. **Methods.** Human islets were isolated using 4 different enzyme mixtures composed of CII plus either intact (CI-115) or degraded CI (CI-100). Blends were administered either with or without NP/CP. Purified islets were cultured for 3 to 4 days before islet quality assessment. **Results.** Whereas using intact CI-115 without NP/CP did not significantly reduce islet yield (3429 ± 631 vs 3087 ± 970 islet equivalent/g, nonsignificant), administration of degraded CI-100 without NP/CP decreased islet yield from 3501 ± 580 to 1312 ± 244 islet equivalent/g ($P < 0.01$), doubled the amount of undigested tissue from 11.8 ± 1.6 to 24.4 ± 1.2% ($P < 0.01$) and triplicated the percentage of trapped islets from 7.7 ± 2.8 to 22.5 ± 3.6% ($P < 0.05$). Islet yield did not vary between supplemented CI-115 and CI-100, but was increased using CI-115 when NP/CP was omitted ($P < 0.05$). A trend toward higher viability and increased secretory insulin response was noted in both CI-100 and CI-115 when NP/CP was not added. **Conclusions.** This study suggests that NP/CP can compensate reduced CI activity. Future attempts to optimize enzyme blends should consider the possibility to increase the proportion of collagenase CI to reduce the need for potentially harmful NPs.

(*Transplantation Direct* 2016;2: e47; doi: 10.1097/TXD.0000000000000552. Published online 15 December 2015.)

Transplantation of isolated human islets offers a promising therapeutic option to restore euglycemia and to normalize pathologic alterations of the protein and lipid

metabolism in patients with type 1 diabetes which is essential to ameliorate progression of nephropathy, retinopathy, and cardiovascular diseases.[1-3] Moreover, islet transplantation is the only minimal invasive procedure to cure hypoglycemia unawareness in prone patients with type 1 diabetes.[4,5]

However, the difficulty to isolate a sufficient islet mass from a single-donor pancreas is the major obstacle for the broad application of clinical islet allotransplantation. Although donor variability and low pancreas quality significantly contribute to the inconsistency of islet isolation outcome,[6-9] reliable enzyme lot-to-lot consistency may be of critical importance for isolation success.[10,11]

Release of islets from within the acinar tissue of the pancreas essentially requires the synergistic interplay between collagenase class I (CI), class II (CII), and clostridial neutral protease (NP) or thermolysin which degrade different collagen structures during pancreas digestion. In this interaction, CII seems to play a major role in pancreas digestion, whereas CI takes the supporting part of collagen degradation.[12,13] Currently, the activity of CII is measured by the Wunsch assay, whereas the CI activity is differentiated by relating the area under the curve of high-performance liquid chromatography–eluted CII protein to the area under the curve of eluted CI protein.[14] As the ratio between CI and CII activity seems to be important for effective islet isolation, any shift within this delicate balance reduces the efficiency of an enzyme blend.[14,15] A significant factor that can alter the proportion of CI within a blend is the degradation of CI. On the basis of previous studies, demonstrating that large amounts of NP diminish

TABLE 1.

Enzyme blending

Blend	n	Collagenase CI, (kDa)	Collagenase CII, (PZ-U/g)	NP (DMC-U/g) (% of PZ-U/g)	CP (BAEE-U/g) (% of PZ-U/g)
CI-115$_s$	6	115	22.7 ± 1.3	1.3 ± 0.1 (5.9 ± 0.2%)	2.2 ± 0.2 (10.0 ± 0.2%)
CI-100$_s$	6	100	22.5 ± 1.7	1.3 ± 0.1 (5.8 ± 0.2%)	2.2 ± 0.2 (9.8 ± 0.3%)
C-115$_n$	6	115	22.3 ± 1.0	0.14 ± 0.01 (0.6 ± 0.0%)	0.32 ± 0.05 (1.4 ± 0.1%)
CI-100$_n$	6	100	22.6 ± 1.0	0.14 ± 0.01 (0.6 ± 0.0%)	0.28 ± 0.08 (1.2 ± 0.3%)

Collagenase class II activity (PZ-U), NP activity (DMC-U), and CP activity (BAEE-U).

PZ-U indicates phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-prolyl-D-arginine; BAEE-U, benzoyl-L-arginine-ethyl-ester.

differences in the efficiency between different ratios of CII-to-CI,[16] we speculated that this discrepancy may be explained by an excessive use of supplementary NP, clostripain (CP) or thermolysin.

The present study was initiated to investigate the relation between NPs and CI to elucidate the interaction and synergism between those proteolytic enzymes in a prospective approach. The aim was to prove our hypothesis that NPs can compensate reduced CI activity to isolate significant amounts of islets from the human pancreas. To be consistent with our previous study about the effect of intact and degraded CI on islet isolation outcome,[17] NP and CP were combined to serve as complementary proteases.

MATERIALS AND METHODS

Islet Isolation

Pancreata were retrieved, after research consent and ethical approval, from 24 human multiorgan donors within a period of 26 months between 2009 and 2011. Once legal consent had been given, pancreata were procured from multiorgan donors with brain death using cold perfusion with University of Wisconsin (UW) solution (ViaSpan; DuPont Pharmaceuticals Ltd., Herts, United Kingdom) or with histidine-tryptophan-ketoglutarate (Custodiol; Köhler Chemie GmbH, Alsbach, Germany). Explanted pancreata were shipped to the central isolation facility (Uppsala, Sweden) in 400 mL of cold UW or Custodiol.

Human islets were isolated as previously described without the intention to transplant.[18] The isolation procedure was always performed by the same isolation team. Briefly, after removal of the duodenum, the main pancreatic duct was cannulated at the distal part of the pancreatic head and manually distended with cold (8°C) Hank balanced salt solution (HBSS; Gibco-Invitrogen AB, Stockholm, Sweden) using a ratio of 1.2 mL/g trimmed pancreas weight. The distension solution was supplemented with collagenase, NP, and CP as shown in Table 1. The different collagenase mixtures were

characterized by the presence of 22 PZ-U/g CII combined with intact or degraded CI isoforms characterized by a molecular weight of 115 kDa (CI-115) or 100 kDa (CI-100), respectively. Two lots of collagenase (LA35/10, 20090005) had to be processed to provide sufficient material of CII as well as intact CI-115 and degraded CI-100 for this study. The CII and CI, either intact or degraded, were recombined to obtain a CII-to-CI ratio of 0.7 as previously described.[17] The collagenase blend CI-115$_s$ and CI-100$_s$ were completed with the same lots of 1.3 dimethyl-casein (DMC)-U/g of NP (lot 20260005) and of 2.2 benzoyl-L-arginine-ethyl-ester-U/g of CP (lot TLA 49/03) or used as nonsupplemented blends (CI-115$_n$, CI-100$_n$) (Table 1). Nonsupplemented blends (CI-115$_n$, CI-100$_n$) contained traces of NP and CP that were approximately 10-fold lower than the amount of proteases in the supplemented groups. The activities of NP and CP were also calculated as relative proportion of CII activity and are shown in brackets in Table 1. All enzymes were manufactured and provided as individual components by Serva/Nordmark Arzneimittel GmbH & Co. KG (Uetersen, Germany). The assignment of the pancreases to the experimental groups was performed in an alternating manner also considering sex and body mass index to facilitate an equal distribution of donor variables between experimental groups.

Pancreas digestion was performed in a 350-mL digestion-filtration device filled with HBSS continuously utilizing a digestion temperature of 37°C.[19] During recirculation, samples were frequently assessed for amount and integrity of released islets. Digested tissue was collected in 250-mL centrifuge tubes prefilled with 25 mL of cold (4°C) newborn calf serum and centrifuged twice for 1 minute at $150 \times g$. After 60 minutes of storage in 250 mL of 1.2-fold concentrated UW (Apoteket AB, Stockholm, Sweden)[20] the digested tissue was centrifuged for 5 minutes at 3000 rpm in a Cobe 2991 cell processor (Gambro, Lakewood, CO) using a continuous hyperosmolaric Ficoll gradient for separation of islets from exocrine tissue.[18] Purified islet fractions were washed twice in HBSS supplemented with 10% newborn calf serum and collected finally in 100 mL of Connaught Medical Research

TABLE 2.

Donor characteristics

Blend	n	Age, y	Sex (Female/Male)	BMI, kg/m^2	CIT, h	Pancreas, g
CI-115$_s$	6	57.7 ± 2.9	4/2	26.3 ± 3.2	11.3 ± 2.3	83.2 ± 8.7
CI-100$_s$	6	53.8 ± 6.2	3/3	24.9 ± 0.4	9.5 ± 1.3	92.3 ± 15.5
C-115$_n$	6	59.5 ± 5.0	3/3	23.2 ± 0.9	11.0 ± 2.0	74.0 ± 4.6
CI-100$_n$	6	54.2 ± 5.0	3/3	24.3 ± 1.6	11.3 ± 1.9	74.7 ± 3.8

BMI, body mass index; CIT, cold ischemia time.

TABLE 3.
Human islet isolation outcome

Blend	n	Recirculation Time, min	Packed Tissue, mL/g	Undigested Tissue, %	Trapped Islets, %	Fragmentation Score (0-3)	Total yield, IE/pancreas	Yield, IE/g	Purity, %
CI-115$_s$	6	22.3 ± 1.2[a]	0.51 ± 0.07	13.8 ± 2.6[a]	4.3 ± 1.3[b]	1.33 ± 0.42	283 225 ± 56 658[a]	3429 ± 631[a]	62.8 ± 9.6
CI-100$_s$	6	21.7 ± 0.8[a]	0.47 ± 0.07	11.8 ± 1.6[b]	7.7 ± 2.8[a]	1.17 ± 0.31	296 558 ± 36 701[a]	3501 ± 580[b]	60.3 ± 6.6
C-115$_n$	6	25.7 ± 2.0	0.40 ± 0.05	21.1 ± 1.1	13.3 ± 1.7	0.83 ± 0.31	222 247 ± 67 512	3087 ± 970[a]	58.5 ± 4.6
CI-100$_n$	6	29.8 ± 1.1	0.58 ± 0.09	24.4 ± 1.2	22.5 ± 3.6	1.67 ± 0.42	96 775 ± 18 860	1312 ± 244	57.7 ± 7.7

IE, islet equivalent.
[a] $P < 0.05$ vs 100$_n$.
[b] $P < 0.01$ vs 100$_n$.

Laboratories 1066 supplemented with 25 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, 1 mM pyruvate, 10 mM nicotinamide (PAA, Pasching, Austria), 10% fetal calf serum, 2.5 mM L-glutamine, 100 U/μg/mL penicillin-streptomycin (Gibco), and 20 μg/mL ciprofloxacin (Bayer, Leverkusen, Germany). Islet culture was performed in a semiclosed culture bag system (Baxter Medical AB, Stockholm, Sweden) incubated in humidified atmosphere for three to four days at 37°C.[21]

Islet Characterization

Subsequent to purification and after culture, yield of islet equivalents was quantified in a digital image analysis-based procedure as previously described in detail.[22] As this quantification procedure does not allow assessment of unpurified islet samples, pre-purification data were not collected for the present study. Islet morphological integrity was categorized using a fragmentation score from 0 (no fragmentation) to 3 (extensive fragmentation). Isolated islets were distinguished from exocrine tissue using insulin-specific dithizone staining (Sigma-Aldrich AB, Stockholm, Sweden).[23]

Viability and in vitro function were assessed after 3 to 4 days of culture at 37°C. Islet viability was measured as membrane integrity using 25 μmol/L of Syto-13 (Invitrogen) and 50 μmol/L of ethidium bromide (Sigma-Aldrich) for staining of viable and dead cells, respectively.[24] The fluorescence of Syto-13 and ethidium bromide was quantified at, respectively, 545 nm and 490 nm using a fluorometric plate reader.

Mitochondrial functional viability was evaluated in triplicate samples of 100 islets measuring the conversion of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Mannheim, Germany) into formazan as previously described.[25] Formazan formation was measured at 490 nm and expressed as optical density per ng DNA. The intracellular DNA content was determined by means of a fluorometric assay (Quant-it; Invitrogen-Molecular Probes, Stockholm, Sweden).

Islet in vitro function was assessed during static glucose incubation of islets precultured for 3 to 4 days at 37°C. Twenty hand-selected islets with an average diameter of 150 to 200 μm were sequentially incubated in duplicate first for 45 minutes in bicarbonate-free Connaught Medical Research Laboratories 1066 (Applichem GmbH, Darmstadt, Germany) supplemented with 2 mmol/L glucose followed by 45 minutes of incubation at 20 mmol/L glucose finally followed by a second 45-minute period at 2 mmol/L glucose. The glucose stimulation index was calculated by dividing the insulin release at 20 mmol/L glucose by the mean of the basal periods. After incubation, islets were recovered and sonified in acid ethanol for subsequent determination of intracellular insulin content.[26] Intracellular insulin content and insulin release were normalized to islet DNA content.

Data Analysis

All statistical analyses were performed using Prism 6.0d for MacIntosh (GraphPad, La Jolla, CA). Analysis of data was carried out by the nonparametric Kruskal Wallis test followed by Dunn correction for multiple comparisons. The data for glucose-stimulated insulin release at low and high

TABLE 4.

Islet quality assessment after culture

Blend	n	Islet Recovery, %	Islet Purity, %	Islet Viability, %	Mitochondrial Activity, OD/µg DNA	Stimulation Index, 20/2 mM	Insulin content, µU/ng DNA
CI-115$_s$	6	66.5 ± 4.6	61.3 ± 10.2	66.3 ± 4.0	9.9 ± 0.7	2.9 ± 0.5	93 ± 20
CI-100$_s$	6	73.6 ± 10.6	57.0 ± 8.5	67.0 ± 6.6	10.3 ± 0.9	2.3 ± 0.6	168 ± 95
C-115$_n$	6	70.2 ± 6.1	51.9 ± 5.2	75.3 ± 1.9	10.6 ± 1.1	2.3 ± 0.2	200 ± 59
CI-100$_n$	6	87.9 ± 8.6	63.5 ± 8.1	72.5 ± 4.7	9.5 ± 0.8	2.3 ± 0.3	208 ± 78

OD, optical density.

glucose concentrations were compared using the Friedman test followed by Dunn correction. Differences were considered significant at P less than 0.05. P values more than 0.05 are termed nonsignificant (NS). Results are expressed as mean ± SEM.

RESULTS

Isolation Outcome

The donor characteristics are presented in Table 2. No significant differences were found between experimental groups regarding age, body mass index, sex, cold ischemia time and trimmed pancreas weight.

When comparing intact collagenase CI-115$_s$ with degraded collagenase CI-100$_s$ no significant differences were found with regard to digestion variables, such as recirculation time, packed tissue volume, proportion of undigested tissue and trapped islets (Table 3) as long as NP/CP were present during digestion. No alterations were also observed with respect to islet fragmentation, purity, total islet yield, and yield per gram trimmed pancreas tissue. In contrast, when NP/CP were absent during digestion, a trend toward a reduced proportion of trapped islets (NS vs CI-100$_n$) and a lower fragmentation score (NS vs CI-100$_n$) was noted when intact collagenase CI-115$_n$ was used for digestion. However, the differences between CI-100$_n$ and CI-115$_n$ regarding islet yield per gram pancreas were significant ($P < 0.05$ vs CI-100$_n$). No effect was found with respect to recirculation time and amount of undigested tissue (Table 3).

The comparison between supplemented collagenase CI-115$_s$ and nonsupplemented collagenase CI-115$_n$ demonstrated a slightly increased proportion of undigested tissue (NS vs CI-115$_s$) and trapped islets (NS vs CI-115$_s$) when NP/CP were not added (Table 3). Total islet yield and islet yield per gram trimmed pancreas were comparable between experimental groups.

Analyzing the isolation outcome obtained by degraded collagenase CI-100 clearly revealed that NP/CP were essentially needed to isolate acceptable islet yields per pancreas ($P < 0.05$ vs CI-100$_n$) or per gram pancreas ($P < 0.01$ vs CI-100$_n$). As shown in Table 3, the low islet yield obtained in the virtual absence of NP/CP correlated with the significantly increased recirculation time ($P < 0.05$ vs CI-100$_s$), doubling of undigested tissue ($P < 0.01$ vs CI-100$_s$) and a 3-fold increased percentage of trapped islets ($P < 0.05$ vs CI-100$_s$). In contrast, none of the digestion parameters or islet yield obtained by using supplemented CI-100$_s$ was significantly altered when compared with nonsupplemented CI-115$_n$, indicating again that the presence of NP/CP can fully compensate the lack of intact CI-115.

Islet Quality Assessment

After 3 to 4 days of culture at 37°C, islets were harvested, and quality assessment was performed. As shown in Table 4, no significant differences were observed between experimental groups in terms of islet recovery, purity, mitochondrial activity, glucose stimulation index, and intracellular insulin content comparing intact with degraded collagenase CI either in the presence (CI-115$_s$ vs CI-100$_s$) or in the virtual absence of NP/CP (CI-115$_n$ vs CI-100$_n$). Also, when the effect of NP/CP on intact (CI-115$_s$ vs. CI-115$_n$) or degraded CI (CI-100$_s$ vs CI-100$_n$) was separately analyzed, no significant variations were observed. Assessment of islet viability indicated that only islets isolated without supplementation of NP/CP had a viability that was above 70% in average. However, this trend did not reach statistical significance.

Islet in vitro function was assessed during sequential incubation at 2, 20, and again 2 mmol/L of glucose. The composition of the different enzyme blends had no significant effect on the glucose stimulation index as shown in Table 4. Nevertheless, islets isolated using NP/CP were characterized by a slightly lower intracellular insulin content compared with the other experimental groups (NS). Islets from any of the experimental groups demonstrated an insulin response toward glucose challenge. As shown in Figure 1, the magnitude of the insulin discharge varied between experimental groups. Islets secreted lower amounts of insulin during glucose challenge when isolated in the presence of NP/CP regardless whether
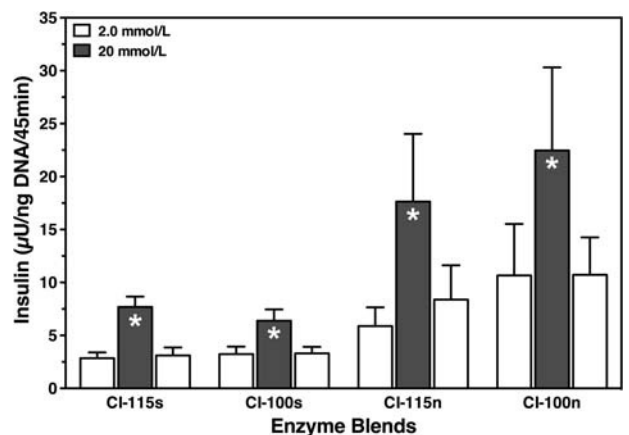

FIGURE 1. Glucose-stimulated insulin release of human islets isolated by means of intact (CI-115) or degraded (CI-100) CI in the presence (CI-115$_s$, CI-100$_s$) or absence (CI-115$_n$, CI-100$_n$) of NP and CP. After culture for 3 to 4 days at 37°C, islets were sequentially incubated for 45 minutes at 2 (white bars), 20 (grey bars), and again 2 mmol/L of glucose (n = 6). Insulin release was normalized to islet DNA content. Dunn test after Friedman test revealed *$P < 0.05$ for insulin secretion at 20 versus 2 mmol/L glucose as indicated.

intact collagenase CI-115$_s$ or degraded collagenase CI-100$_s$ was used for pancreas digestion (NS).

DISCUSSION

Previous and recent studies in rats clearly suggested that the fast and effective release of islets from within the acinar tissue essentially requires the presence of collagenase CI, CII, and NPs.[12,13,27] The balance between CI and CII appears to be critical for efficient islet release[14] but can significantly vary from lot to lot[28] and from product to product.[29] An important factor that changes the proportion of CI activity is the cleavage of 1 of the 2 collagen-binding domains from the CI molecule presumably occuring during the enzyme manufacturing process.[30] The loss of this collagen-binding domain does not only reduce the molecular weight of CI from 115 kDa to 100 kDa but also correlates with a 90% reduction of the specific collagen-degrading activity of CI and the reduced efficiency to successfully separate islets from the human pancreatic acinar tissue.[31] However, an initial prospective study could not demonstrate any difference in human islet isolation outcome using either degraded CI-100 or intact CI-115.[17] The present finding, that it is possible to successfully isolate islets from human pancreata with strongly reduced CI activity is compatible with the observation that NP can compensate a deficiency in CI activity to digest the peri-insular extracellular matrix in rat pancreata.[16] Vice versa, when intact and fully active CI was used for human pancreas digestion, significant numbers of islets were isolated in the present study even in the virtual absence of NPs. These results may also explain why the amount of NPs used for complete human pancreas digestion varies enormously between different isolation centres. Unfortunately, we cannot present data collected prepurification to quantify the loss of acinar-embbeded islets during purification.

When the Wunsch assay is used to determine collagenase activity, degraded CI has a similar collagenase-specific activity compared to nondegraded CI. This corresponds to the daily practice in most islet isolation facilities where usually 1 vial of collagenase, equivalent to approximately 2000 to 2200 PZ-U, is used to digest a human pancreas. Nevertheless, the Wunsch assay mainly determines the CII activity[32] and does not reflect the real collagen-degrading activity of CI when compared to more sensitive techniques.[30,31] The variability among different collagenase products is illustrated by the variability in the amount of NPs that is needed for effective collagenase supplementation in different isolation centers. Human pancreas digestion protocols reveal a wide range of using NP in a range from 50 DMC-U[33] to 400 DMC-U[31] for collagenase supplementation. This indicates that the extensive use of supplementary NP, CP, or thermolysin diminishes differences in various collagenase blends particularly regarding the proportion of intact CI.[16,17] It also explains why protocols for the enzymatic dissociation of human pancreata could not be standardized so far.

In the present study, the use of degraded CI-100, that is, the absence of fully active CI-115 prolonged recirculation time, increased percentage of trapped islets, and reduced islet yield when NP/CP were virtually absent during the digestion process. In contrast, the addition of these complementary proteases completely diminished the differences between CI-100 and CI-115, providing nearly identical results regarding isolation outcome. None of the comparisons between

supplemented CI-100$_s$ and either supplemented CI-115$_n$ or nonsupplemented CI-115$_s$ revealed an extension of the digestion phase or a significant reduction of islet yield.

To draw the conclusion that the addition of large amounts of NPs improves the use of different enzyme blends may nevertheless be misleading, considering the potential toxicity of NPs for islet viability and morphological integrity.[16,34-36] In the present study, a trend toward slightly decreased viability was observed when NP/CP were supplemented. In accordance, this trend was also found analyzing the in vitro function of isolated islets. Islets released lower amounts of insulin after glucose challenge when isolated in the presence of NP/CP. In contrast to previous studies, harmful effects of degraded CI-100 on morphological and functional integrity of isolated islets could not be confirmed in the present study.[37]

On the other hand, the data of the present study clearly imply that NPs can be completely avoided if fully active CI is provided in sufficient amounts. In agreement with our previous experiments in rats,[16] the Edmonton group demonstrated an improvement of human islet isolation outcome when CI represent the majority of collagenase protein.[38] Moreover, the retrospective analysis of 163 human islet isolations revealed that final islet purity significantly correlates with CI activity.[32]

In summary, for the first time, it is demonstrated that significant numbers of islets can be released from the human pancreas with significantly reduced CI activity provided that NP and CP are present during pancreas digestion. On the contrary, we show in older donors that significant islet yields can be isolated in the virtual absence of NPs as long as a sufficient proportion of fully active CI is used for pancreas digestion. These observations imply that NPs can compensate reduced collagenase CI activity to digest human pancreata for efficient islet release. Future efforts to optimize enzyme blends should consider the possibility to increase the proportion of collagenase CI to reduce the need for potentially harmful NPs. The findings of the present study may be a step forward to establish a standardized human islet isolation protocol.

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