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Prevention by rolipram of concanavalin A-induced T-cell-dependent hepatitis in mice

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Abstract

Rolipram is a type IV phosphodiesterase inhibitor endowed with powerful immunomodulatory properties. In this study, we evaluated the effects of this drug on the development of the T-cell-mediated hepatitis inducible in mice by concanavalin A. The results indicated that prophylactic treatment with either 5 or 10 mg/kg rolipram injected intraperitoneally 24 h and 1 h prior to intravenous (i.v.) challenge with 20 mg/kg concanavalin A successfully ameliorated serological and histological signs of liver damage, so that the treated mice showed lower transaminase levels in the plasma and milder mononuclear cell infiltration of the liver as compared to vehicle-treated controls. Moreover, this effect was associated with profound modifications of circulating levels of cytokines released after concanavalin A injection, with the blood levels of interferon-γ and tumor necrosis factor-α being significantly lower and those of interleukin-10 higher than those of the control mice. In particular, the increased blood levels of interleukin-10 might play an important role in the anti-hepatitic effects of rolipram as coadministering this compound with anti-interleukin-10 monoclonal antibody significantly reduced its anti-inflammatory action. These results suggest that rolipram may be useful in the clinical setting for the treatment of cell-mediated immunoinflammatory diseases such as immunoinflammatory hepatitis.

Keywords: Concanavalin A; Cytokine; Hepatitis; Immunotherapy; Interleukin-10; Rolipram

1. Introduction

An immunoinflammatory model of hepatitis with characteristics similar to those human autoimmune hepatitis has recently been described that is inducible in mice by one single intravenous (i.v.) injection of concanavalin A (Tiegs et al., 1992; Mizuhara et al., 1994; Gantner et al., 1995). The hepatic lesions occur shortly (8–24 h) after concanavalin A application and are histologically characterized by infiltration of the liver by mononuclear cells and granulocytes with necrotic areas and apoptotic cell death of the hepatocytes associated with marked elevation of hepatic enzymes (Tiegs et al., 1992; Mizuhara et al., 1994; Gantner et al., 1995). The disease appears to be T-cell/macrophage-dependent as it cannot be induced in nude athymic mice lacking immunocompetent T-cells, is prevented by anti-CD4 monoclonal antibody, silica particles which inhibit macrophage functions, or drugs capable of down-regulating macrophage and/or T-cell activities such as cyclosporin A, tacronimus, and sodium fusidate (Tiegs et al., 1992; Gantner et al., 1995; Nicoletti et al., 1997a,b). The central role played from the cytokine network in the pathogenesis of this experimental model is highlighted by the beneficial effects of anti-interferon-γ (Kusters et al., 1996; Mizuhara et al., 1996), anti-tumor necrosis factor-α (Mizuhara et al., 1994; Gantner et al., 1995) and anti-interleukin-4 antibodies (Toyabe et al., 1997), the resistance to the syndrome of interferon-γ knockout mice (Tagawa et al., 1997), and the anti-hepatitic

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effects exerted by exogenously administered interleukin-6 (Mizuhara et al., 1994; Mizuhara et al., 1996) and interleukin-10 (Louis et al., 1997). Results of these studies also suggest that drugs capable of suppressing the production of interleukin-4, tumor necrosis factor-α and interferon-γ or of up-regulating the production of interleukin-6 or interleukin-10 may be suitable candidates for the treatment of concanavalin A-induced hepatitis.

Rolipram is a type IV phosphodiesterase inhibitor which has recently been shown to possess powerful immunomodulatory properties including modulation of cytokine release and inhibition of cellular proliferation, motility, chemotaxis and migration to sites of inflammation (Torphy and Undem, 1991; Semmler et al., 1993; Siegmund et al., 1997; Eigler et al., 1998). Accordingly, rolipram has been reported to ameliorate immunoinflammatory diseases in several experimental models (Genain et al., 1995; Sekut et al., 1995; Sommer et al., 1995; Jung et al., 1996; Sommer et al., 1997; Nyman et al., 1997; Ross et al., 1997; Liang et al., 1998).

These observations prompted us to evaluate the effects of rolipram in concanavalin A-induced hepatitis. The results indicated that prophylactic, but not therapeutic, treatment with this compound prevented clinical and serological signs of concanavalin A-induced hepatic injury which were associated with reduced blood levels of tumor necrosis factor-α and interferon-γ along with an increase in interleukin-10. The latter effect seemed to be centrally involved in the anti-hepatitic effects of rolipram as block of *endogenous* interleukin-10 with a monoclonal antibody reduced, but did not abolish, the immunomodulatory efficacy of the drug at both the clinical and seroimmunological levels.

2. Materials and methods

2.1. Mice and hepatitis induction

Six- to eight-week old male Naval Medical Research Institute (NMRI) albino mice were purchased from Charles River (Calco, Italy). The mice were kept under standard laboratory conditions (non-specific pathogen-free) at 24°C with free access to food and water. The food was withdrawn 16 h prior to the experiments. The mice were divided into three experimental groups and were challenged with 20 mg/kg concanavalin A (Sigma, St. Louis, MO). Concanavalin A was dissolved in sterile phosphate buffered saline (PBS) and injected via the tail vein. The groups were treated intraperitoneally either with the vehicle used to dissolve rolipram, consisting of PBS containing cremophor at a concentration 10% (w/v), or with rolipram 4-(3-cyclopentyloxy-4-methoxyphenil)-2-pyrrolidone (Sigma) according to the experimental design shown in Table 1. An additional control group consisted of untreated mice not challenged with concanavalin A (Table 1).

Table 1
The effects of rolipram on concanavalin A-induced hepatitis

Treatment (*n*)	Dose	Concanavalin A	Alanine aminotransferase (U/l)	*P*
Nil (10)	–	–	56±3	
Vehicle (16)	200 μl	+	8906±217	Control
Rolipram (19)	5 mg/kg	+	146±14	< 0.0001
Rolipram (15)	10 mg/kg	+	89±2	< 0.0001

Rolipram, dissolved in PBS containing cremophor (Sigma) at a concentration of 10% (w/v) was injected intraperitoneally into the mice 24 and 1 h prior to i.v. challenge with 20 mg/kg concanavalin A. Control mice were treated under similar conditions with vehicle only, and served as control group. A total of 8 h after concanavalin A application the mice were killed and blood samples were collected from individual mice for alanine aminotransferase measurement (in U/l). For statistical analysis each group was compared to vehicle-treated control animals. The results of three independent experiments are shown.

2.2. Anti-interleukin-10 monoclonal antibody

The SXC.1 rat immunoglobulin (IgG) M anti-mouse interleukin-10 monoclonal antibody was produced and purified as previously described (Moosmann et al., 1990; Nicoletti et al., 1997a,b). Irrelevant rat IgG (Sigma, St. Louis, MO) was used as control. Both anti-interleukin-10 monoclonal antibody and irrelevant rat IgG were diluted in sterile pyrogen-free PBS and injected intraperitoneally to the mice in a final volume of 1 ml. The endotoxin levels of all these preparations were < 15 pg/ml as determined by a *Limulus* amoebocyte lysate assay (Whittaker M.A., Bioproducts, Walkersville, MD).

2.3. Assay for plasma transaminase activity

Plasma alanine aminotransferase activity was determined with a standard photometric assay using a bichromatic analyzer.

2.4. Cytokine measurements

Plasma samples from individual mice were collected for the measurement of interferon-γ, tumor necrosis factor-α and interleukin-10, using solid phase enzyme-linked immunoassay (ELISA) kits for the detection of mouse cytokines provided from Genzyme (Cambridge MA). Samples were run in duplicate according to the manufacturer's instructions. The lower limit of sensitivity of the assays was 2 pg/ml for interferon-γ, 10 pg/ml for interleukin-10 and 15 pg/ml for tumor necrosis factor-α. To calculate mean values, samples with cytokine values below the level of detection were assigned the limit of sensitivity of the assay as theoretical values.

2.5. Histological examinations

8 h after concanavalin A injection, the livers were removed, fixed in 10% formalin, embedded in paraffin, sliced into 5-μm sections and stained with hematoxylin and eosin for histological examinations at 125-fold magnification. This was performed by two observers unaware of the treatment of the mice.

2.6. Calculation of data

Results are expressed as mean values ± S.E.M. Statistical analysis was performed by analysis of variance (ANOVA).

3. Results

3.1. Prophylactic but not therapeutic treatment with rolipram prevented serological and histological signs of concanavalin A-induced hepatic injury

Four of 20 (20%) concanavalin A/PBS-treated control mice, and one of 20 (5%) of those challenged with concanavalin A and treated with 5 mg/kg rolipram died before the time to killing and thus were not considered for serological and histological analyses. As expected, and in agreement with previous studies (Tiegs et al., 1992; Mizuhara et al., 1994; Gantner et al., 1995; Kusters et al., 1996; Mizuhara et al., 1996; Louis et al., 1997; Nicoletti et

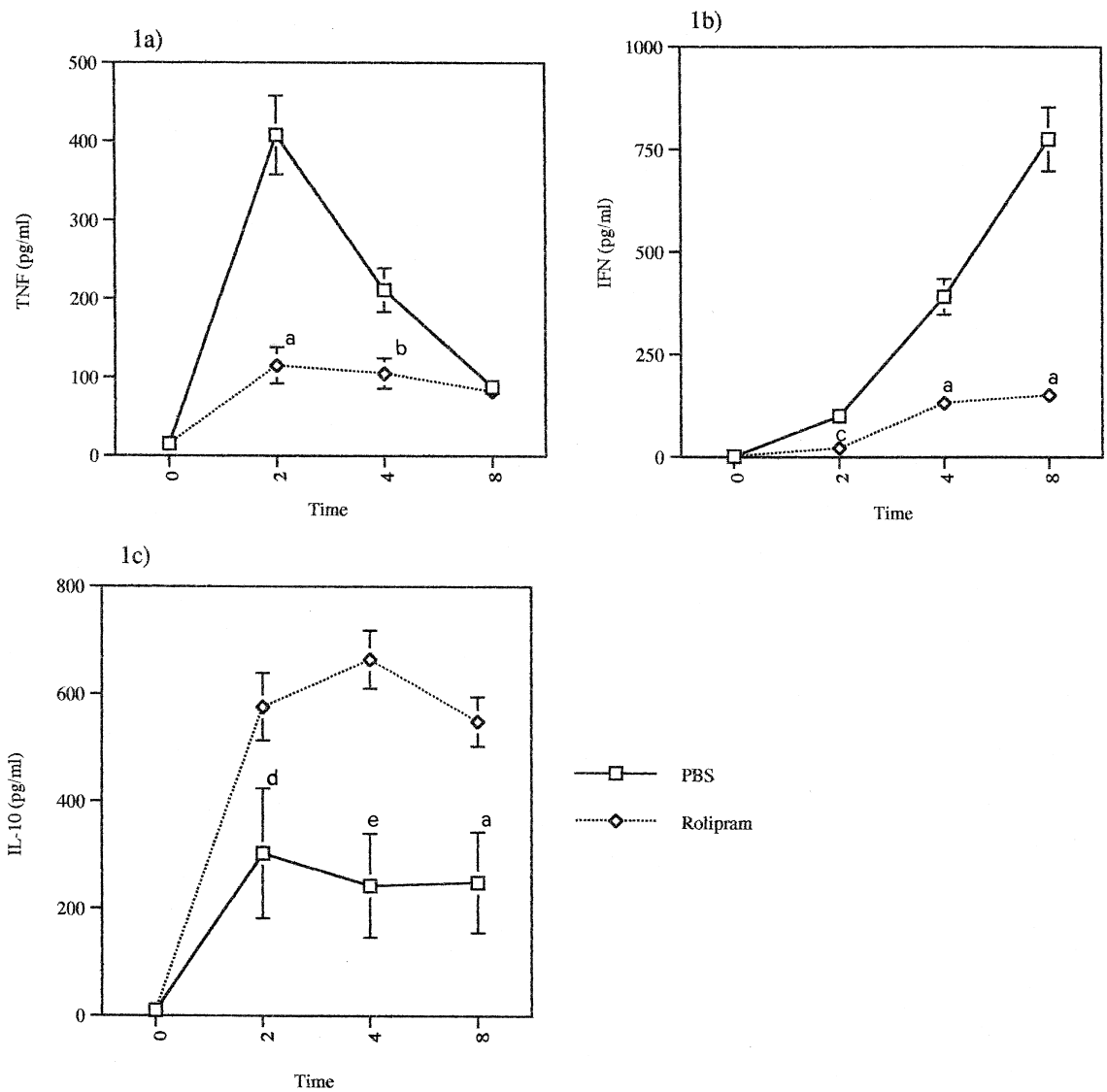

Fig. 1. (a–c) Modulation by rolipram of concanavalin A-induced cytokine release. Seven mice from each group were killed before (TO) and 2, 4 and 8 h after injection of concanavalin A, and plasma samples from individual mice were used for cytokine measurements. All the mice were injected i.p. with either rolipram (10 mg/kg) or PBS, 24 and 1 h before concanavalin A challenge. Data are shown as means ± S.E.M. For statistical analysis each group was compared to the vehicle-treated control group. (a) $P = 0.0001$; (b) $P = 0.01$; (c) $P = 0.02$; (d) $P = 0.004$; (e) $P < 0.0001$, by ANOVA.

al., 1997a; Tagawa et al., 1997; Toyabe et al., 1997), signs of liver damage were found after killing in all the control mice that had been injected with concanavalin A and treated with PBS. The signs consisted of marked elevations of alanine aminotransferase in the plasma (see Table 1) and severe lobular infiltration with neutrophil granulocytes, lymphocytes and monocytes (data not shown). The inflammatory process was also detected both in the portal areas and around the central veins, and diffuse hepatocytic necrosis which contained neutrophil granulocytes was also observed (data not shown). In contrast, pretreatment with rolipram successfully prevented the biochemical and histological signs of hepatic injury, regardless of the dose used (Table 1 and data not shown). However, delaying rolipram treatment (10 mg/kg) until 2 h after concanavalin A injection failed to provide serological and histological protection against concanavalin A-induced hepatitis, and these mice showed a clinical and histological picture similar to that of the control group (data not shown).

3.2. The blood of rolipram-treated mice contained less tumor necrosis factor-α and interferon-γ and more interleukin-10 than did the blood of control mice

To evaluate whether the beneficial effects of rolipram on the development of hepatitis were associated with modulation of the cytokine release pattern induced by concanavalin A, we studied the effect of the treatment on the blood levels of tumor necrosis factor-α, interferon-γ and interleukin-10. Two groups of mice treated with either PBS or rolipram (10 mg/kg) 24 h and 1 h prior to concanavalin A-application, were killed before concanavalin A-injection (T0) and 2, 4, and 8 h thereafter. As shown in Fig. 1a–c, the blood levels of tumor necrosis factor-α, interferon-γ and interleukin-10 were all below the limit of sensitivity of the assay at T0. However, after concanavalin A-challenge, these cytokines were massively released with different kinetics into the circulation, more rapidly (+2 h, +4 h after concanavalin A challenge) for tumor necrosis factor-α and interleukin-10, and more slowly for interferon-γ (see Fig. 1a–c). Rolipram-treated mice showed profound modifications of this cytokine pattern, in that their circulating levels of tumor necrosis factor-α and interferon-γ were lower and those of interleukin-10 significantly higher than those of the control mice (Fig. 1a–c).

3.3. The anti-hepatitic effects of rolipram were partly reversed by anti-interleukin-10 monoclonal antibody treatment

To evaluate whether the increased blood levels of interleukin-10 were involved in the anti-hepatitic effects of rolipram, we aimed at counteracting the bioavailability and action of *endogenous* interleukin-10 in rolipram-treated mice by intraperitoneal injection with a neutralizing anti-interleukin-10 monoclonal antibody. The control groups consisted of concanavalin A-challenged mice treated intraperitoneally with either anti-interleukin-10 monoclonal antibody or irrelevant rat IgG. As expected, clinical and serological signs of hepatitis similar to those previously found in concanavalin A-challenged mice treated with PBS (Table 1) were found 8 h after concanavalin A-injection in the group of mice treated with irrelevant rat IgG. In agreement with results of a previous study, pretreatment of the mice with anti-interleukin-10 monoclonal antibody worsened the course of the disease, so that the mice from this group exhibited higher plasma levels of alanine aminotransferase and more severe histological signs of liver damage than the Ig-treated mice (Table 2 and data not shown). Confirming our earlier results, rolipram prophylaxis again prevented the hepatitic effects of concanavalin A. However, the action of the drug was significantly diminished, though not abolished, when the treatment was combined with SXC.1 anti-interleukin-10 monoclonal antibody: the mice so-treated exhibited alanine aminotransferase plasma levels and histological signs of hepatitis with values intermediate between those for the group of mice treated with irrelevant rat IgG and those for the mice treated with rolipram alone (Table 2 and data not shown). There was, however, a highly significant difference ($P = 0.0001$) in alanine aminotransferase values between the group of mice treated with rolipram alone and that treated with rolipram plus anti-interleukin-10 monoclonal antibody.

Table 2
Anti-interleukin-10 monoclonal antibody treatment does not reverse the anti-hepatitic effects of rolipram

Treatment	Dose	Concanavalin A	Alanine aminotransferase	*P*
Irrelevant rat IgG	2 mg	+	8322±250	Control
SXC.1	2 mg	+	18.986±156	< 0.0001
Rolipram	10 mg/kg	+	114±9	< 0.0001
Rolipram +SXC.1	10 mg/kg +2 mg	+	2114±523	= 0.0001

Irrelevant rat IgG and SXC.1 anti-interleukin-10 monoclonal antibody were administered 2 h prior to concanavalin A-injection, and rolipram was administered as above 24 and 1 h prior to challenge. The mice treated simultaneously with rolipram and SXC.1 monoclonal antibody received rolipram as above, plus two injections with anti-interleukin-10 monoclonal antibody, each given 24 and 2 h prior to rolipram. For statistical analysis, each group was compared to the mice treated with irrelevant rat IgG. The results of three independent experiments are shown. Each group consisted of 15 mice. However, one mouse that died in the group treated with irrelevant rat IgG and two in the group treated with anti-interleukin-10 monoclonal antibody were not considered for alanine aminotransferase measurement (in U/l).

4. Discussion

We have shown for the first time that prophylactic treatment with rolipram successfully prevented serological

and histological signs of concanavalin A-induced hepatitis in mice. The blood levels of macrophage and/or T-cell-derived cytokines such as tumor necrosis factor-α, interferon-γ, and interleukin-10, which are released in the circulation of the mice after concanavalin A injection and may be pathogenetically related to the inflammatory syndrome (Tiegs et al., 1992; Mizuhara et al., 1994; Gantner et al., 1995; Kusters et al., 1996; Mizuhara et al., 1996; Louis et al., 1997; Nicoletti et al., 1997a; Tagawa et al., 1997; Toyabe et al., 1997), were also profoundly modified by the drug. Relative to that of the control mice, the blood of rolipram-treated mice contained less tumor necrosis factor-α and interferon-γ and more interleukin-10. Because tumor necrosis factor-α and interferon-γ play a pathogenic role in the development of concanavalin A-induced hepatitis (Mizuhara et al., 1994; Gantner et al., 1995; Kusters et al., 1996; Mizuhara et al., 1996), while interleukin-10 exerts an anti-inflammatory and protective action (Louis et al., 1997), these data suggested that the above modifications in the circulating levels of these cytokines might have contributed to the anti-inflammatory effects of rolipram. This view was further substantiated by the observation that block of *endogenous* interleukin-10 with a monoclonal antibody substantially reduced the anti-hepatitic effects of rolipram. The precise mode of action by which anti-interleukin-10 monoclonal antibody treatment partly counteracted the protective effects of rolipram has not been investigated. However, our and other studies (Ishida et al., 1993; Ishida et al., 1994; Nicoletti et al., 1997b) have shown that anti-interleukin-10 treatment modifies the immunological status of the mice, leading to enhanced production of type 1 proinflammatory cytokines implicated in the pathogenesis of concanavalin A-induced hepatitis such as interferon-γ and tumor necrosis factor-α. This, together with diminished availability of *endogenous* interleukin-10 caused by the monoclonal antibody may have reduced the anti-inflammatory action of rolipram in this model. That the anti-hepatitic effects of the drug were not completely abolished by anti-interleukin-10 monoclonal antibody is consistent with results of other studies showing that suppression of tumor necrosis factor synthesis by rolipram is not mediated by an augmented interleukin-10 production (Eigler et al., 1998; Haskò et al., 1998). It is thus possible that a significant inhibition of tumor necrosis factor-α release was provided by rolipram even in the presence of anti-interleukin-10 monoclonal antibody which may have allowed the slight anti-hepatitic action to be maintained.

The present observations extend to the concanavalin A-induced model of hepatitis the beneficial effects observed with rolipram in other cell-mediated immunoinflammatory diseases in animal models of acute and chronic inflammation, multiple sclerosis, rheumatoid arthritis and insulin-dependent diabetes mellitus (Genain et al., 1995; Sekut et al., 1995; Sommer et al., 1995; Jung et al., 1996; Nyman et al., 1997; Ross et al., 1997; Sommer et al., 1997; Liang et al., 1998). In particular, in rodent experimental allergic encephalomyelitis (Sommer et al., 1995; Jung et al., 1996), type II collagen-induced arthritis (Ross et al., 1997) and insulin-dependent diabetes mellitus (Liang et al., 1998), the drug modulated cytokine secretion in a manner very similar to that we presently observed, inhibiting tumor necrosis factor-α, and interferon-γ (and interleukin-12) and enhancing interleukin-10 production. Because interleukin-10 is capable of down-regulating cell-mediated proinflammatory responses mostly through its down-regulatory effects on macrophage function (see the work of Moore et al., 1993 for a review), all these data suggest a general mechanism of action for the anti-inflammatory effect of rolipram that may be, at least partly, based on its capacity to up-regulate the production of this cytokine.

Similarly to the case of Lewis rat experimental allergic encephalomyelitis, where the preventive, but not therapeutic, application of rolipram ameliorates the course of the disease (Jung et al., 1996), rolipram also did not exert therapeutic effects when administered to the mice 2 h after concanavalin A. This is in contrast to the reported therapeutic effects of chlorpromazine (another interleukin-10 inducer) in this model (Ikeda et al., 1997), probably due to different modes of action of the two compounds in this experimental setting. The reason for the lack of a therapeutic effect of rolipram in this model is not known. As a massive release of tumor necrosis factor-α in the blood occurs as early as 1–2 h after concanavalin A application, it could be that the hepatitogenic pathways cannot be modulated by rolipram once the initial burst of tumor necrosis factor-α production has taken place. Caution should however be exercised in generalizing from this experimental observation that rolipram cannot be used in patients with established immunoinflammatory hepatitis. The differences between humans and mice must obviously be taken into account, as should the observation that even drugs of known clinical utility such as cyclosporin A fail to exert therapeutical efficacy as 'therapeutic' regimen in concanavalin A-induced hepatitis (Ikeda et al., 1997).

Our present data further highlight the regulatory role of *endogenous* interleukin-10 in this experimental model. In particular, along with the disease-exacerbating effects of anti-interleukin-10 monoclonal antibody, we also confirmed the increased blood levels of interleukin-10 during concanavalin A-induced hepatitis, previously reported by Louis et al. (1997), and which may represent a homeostatic anti-inflammatory attempt aimed at counteracting the ongoing cell-mediated attack against the hepatocytes. That Ikeda et al. (1997) did not find augmented circulating levels of interleukin-10 during the course of concanavalin A-induced hepatitis is difficult to explain as these authors used the same ELISA kit as used by ourselves and Louis et al. (1997) to measure interleukin-10. These differences could be related to the use of BALB/c mice by these authors while we and Louis et al. used NMRI and

C3H/HeJ mice, respectively. In fact, the production of cytokines is known to be genetically influenced by the major histocompatibility complex haplotype of the mouse strain considered, which might modulate the susceptibility or resistance to several immunoinflammatory diseases (Doth et al., 1997).

In conclusion, our present observation that rolipram may successfully prevent concanavalin A-induced hepatitis further emphasizes the potential immunomodulatory properties of this drug, the use of which should also be considered for the treatment of cell-mediated immunoinflammatory diseases in humans, such as autoimmune hepatitis.

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