CHARACTERIZATION OF BINDING AND BIOLOGICAL EFFECTS OF MONOCLONAL ANTIBODIES AGAINST A HUMAN TUMOR NECROSIS FACTOR RECEPTOR

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TNF-α, also called cachectin, and TNF-β, also called lymphotoxin, are pleiotropic cytokines that can mediate a wide variety of biological effects. TNF-α is mostly produced by activated macrophages, whereas TNF-β is produced by activated lymphocytes (1, 2). These two cytokines are similar both in amino acid sequence and in inducing similar biological effects (3, 4). TNF-α induces cytotoxicity in various tumor cells (5), stimulates the growth of fibroblasts (6), and regulates the functions of endothelial cells (7), neutrophils (8), T cells (9), B cells (10), as well as NK/LAK-cells (11). TNF-α interacts with a variety of target cells by first binding to a specific membrane receptor that can be upregulated by treatment with IFN-γ (12). The biochemical characteristics of the TNF receptor are still unclear. However, crosslinking experiments with radiolabeled TNF-α indicated the presence of at least two polypeptides associated with the TNF receptor (13, 14). Recently, mAbs against human TNF receptor proteins purified from HL-60 cells were prepared (Brockhaus, M., and H. Loetscher, manuscript submitted for publication). Three of these antibodies (htr-1, htr-5, and htr-9) were selected for further studies. In this article we present results on their binding pattern to U937 cells and biological effects on U937, Fs4 fibroblasts, and endothelial cells. The results demonstrate that htr-9 binds to U937 cells and that this binding can be blocked by pretreating the cells with TNF-α or TNF-β, whereas htr-5 binding was inhibited by TNF-α but only to a minimal extent by TNF-β pretreatment. Furthermore, htr-1 and htr-9, but not htr-5, were found to mimic TNF-α action on U937 cells, Fs4 fibroblasts, and endothelial cells. The data indicate that these antibodies recognize structures of a TNF receptor that are involved in signal transduction and structures that differentiate between TNF-α and TNF-β binding to target cells.

Materials and Methods

Cytokines and Antibodies. tTNF-α and tTNF-β were generously provided by Genentech Inc. (South San Francisco, CA) and had specific activities of 7.6 x 10⁷ U/mg and 10 x 10⁷ U/mg, respectively, as determined in the L-M bioassay (15). The generation of mAbs htr-1, htr-5, and htr-9 against human TNF receptor proteins is described elsewhere (Brockhaus, M., and...
H. Loetscher, manuscript submitted for publication). The htr-5 and htr-9 are both of IgGl isotype and htr-1 is an IgM antibody. These antibodies were affinity purified from hybridoma supernatants by using rabbit anti-mouse Ig linked to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). rTNF-α and affinity-purified htr-5 and htr-9 were for some experiments labeled with biotin as previously described (16). The pfa-11, an IgGl mouse mAb that recognizes human platelet-derived growth factor A chain, was used as a control. Streptavidin-phycoerythrin was purchased from Becton Dickinson & Co., Mountain View, CA.

**Flow Cytometric Quantification of TNF Receptor Antibody Binding and rTNF-α Binding**

U937 and HL-60 cells (American Type Culture Collection, [ATCC], Rockville, MD) were grown in RPMI 1640 (Gibco Laboratories, Paisley, Scotland) supplemented with 10% heat-inactivated FCS (Gibco), 2 mM l-glutamine, and 40 μg/ml gentamicin (complete RPMI 1640). Binding of TNF receptor antibodies or rTNF-α to cells was done by adding 10 μg/ml of biotinylated antibodies or 50 ng/ml of biotinylated rTNF-α (BrTNF-α) diluted in PBS with 0.1% BSA (Sigma Chemical Co., St. Louis, MO) to 10⁶ cells for 45 min at 0-4°C. The cells were then washed twice in PBS/BSA and were stained with 20 μl of streptavidin-phycoerythrin for 45 min at 0-4°C. The cells were subsequently washed once in PBS and fixed in 2% formalin (Merck, Darmstadt, Federal Republic of Germany) and stored at 0-4°C until they were analyzed using a FACScan flow cytometer (Becton Dickinson & Co.). Blocking experiments were done by pretreating cells with one of the following reagents: 50 μg/ml of rTNF-α, 50 μg/ml of rTNF-β, 10 μg/ml of htr-9 or htr-5 for 45 min at 0-4°C before addition of the biotinylated reagent. For flow cytometric analysis 5 x 10⁴ cells were acquired by list mode and measurements were performed on a single cell basis and were displayed as frequency distribution histograms.

**Cytotoxicity Assay.** About 3 x 10⁶ U937 cells were labeled with 300 μCi Na₂⁵¹CrO₄ (Amersham, Buckinghamshire, UK) for 90 min at 37°C, followed by two washes in RPMI 1640 and one wash in complete RPMI 1640. 100 μl of labeled U937 cells (10⁵ cells/ml) in complete medium were added to flat-bottomed microtiter plates (Costar, Cambridge, MA) together with 100 μl of different dilutions of TNF receptor antibodies or rTNF-α. After 20 h of incubation at 37°C, 100 μl of supernatants were harvested and their radioactivity was measured in an LKB Wallac 1270 gamma counter (Turku, Finland). Percent specific lysis was calculated as follows: percent specific lysis = 100 x [(A-B)/(C-B)], where A represents cpm from test supernatants; B represents cpm from supernatants with target cells alone (spontaneous release); and C represents cpm after lysis of target cells with 0.25% SDS (maximum release). Results are presented as the mean ± SE of triplicate cultures. Spontaneous release from U937 cells alone was <25% for all experiments.

**Thymidine Incorporation Assay.** Fe₄ fibroblasts (generously provided by Dr. J. Višek, New York University, New York, NY) were grown in complete RPMI 1640. 100 μl of 10⁵ cells/ml were dispensed in triplicate wells in flat-bottomed microtiter plates (Costar) together with 100 μl of different dilutions of TNF receptor antibodies or rTNF-α. After 72 h of incubation 1 μCi methyl-[³²H]thymidine (Amersham) was added to each well 4 h before harvesting the cultures with a TiterTec cell harvester (Flow Laboratories, Ayrshire, UK). Radioactivity was measured by liquid scintillation counting in an LKB Wallac 1215 beta counter. Results are presented as cpm ± SE of triplicate cultures.

**Human Endothelial Cell Cultures and Assays.** Human endothelial cells (HEC) were obtained by collagenase (Sigma Chemical Co.) treatment of human umbilical cord veins as described (17). The identity of HEC was confirmed as detailed in a previous report (18). The cells were seeded at uniform density (0.15 x 10⁶/well) in 24-well plates (Costar) using complete RPMI 1640. For the induction of IL-6, cultures of confluent HEC monolayers were incubated with or without the addition of different concentrations of TNF receptor antibodies or rTNF-α. After 24 h of incubation the supernatants were collected and tested for IL-6 activity as detailed previously (18). For adhesion of HL-60 cells, cultures of confluent HEC monolayers were subjected to treatment with TNF receptor antibodies or rTNF-α for 5-6 h at 37°C. HEC monolayers were then washed twice with HBSS (Gibco) and each culture received 250 μl of complete RPMI 1640 containing 0.5 x 10⁵ [⁵¹Cr] HL-60 (HL-60 were labeled with [⁵¹Cr] and washed as described above). After 1 h of incubation nonadherent [⁵¹Cr] HL-60 cells were removed and after four washes with HBSS HEC cultures received 250 μl of 1% Triton-100
(Sigma Chemical Co.) and were further incubated for 1 h to lyse the cells. The radioactivity was determined by counting aliquots of cell lysate using LKB Wallac 1270 gamma counter. Maximum count was obtained from 0.1% Triton-100–treated 0.5 × 10^5 ^{51}Cr HL-60 cells. The mean cpm as a reflection of ^{51}Cr HL-60 adherence is expressed as percentage of the mean maximum cpm.

Results

**Specificity of Binding of TNF Receptor Antibodies to U937 Cells.** Experiments were carried out to quantify the binding of htr-9 and htr-5 to U937 cells and to see if rTNF-α or rTNF-β could inhibit the binding of these TNF receptor antibodies. The results are shown in Fig. 1 and demonstrate that biotinylated (B) htr-9 significantly bound to U937 cells and that pretreatment of the cells with either rTNF-α or rTNF-β reduced the binding almost to background level (Fig. 1, A and B). These data indicate that htr-9 recognizes an epitope of a TNF receptor that is common for TNF-α and TNF-β binding. Similar experiments were also performed with htr-5. As shown in Fig. 1, C and D, pretreatment of the cells with rTNF-α reduced the binding of Bhtr-5 to background levels, however, pretreatment with rTNF-β only minimally

![Flow cytometric analysis of U937 cells stained with 10 µg/ml of Bhtr-9 (A, B) or 10 µg/ml of Bhtr-5 (C, D). Where indicated, U937 cells were pretreated with 50 µg/ml of rTNF-α (A, C) or 50 µg/ml rTNF-β (B, D) before addition of the biotinylated antibodies. The dotted histograms represent the background staining obtained with the streptavidin-phycocerythrin reagent alone.](attachment:image.png)
reduced Bhtr-5 binding. These results suggest that htr-5 recognizes an epitope of a TNF receptor that is mainly involved in binding of TNF-α but to a lesser extent TNF-β. The structural differences among the epitopes of the TNF receptor as recognized by htr-9 and htr-5 were further studied in another set of blocking experiments. U937 cells were pretreated with htr-1 or htr-5 antibodies and thereafter stained with Bhtr-9 before they were analyzed by flow cytometry. The results are shown in Fig. 2, A and B and demonstrate that htr-1 and htr-5 inhibited the binding of Bhtr-9. These data indicate that htr-9 recognizes an epitope of a TNF receptor that is overlapping with epitopes recognized by htr-5 and htr-1. A similar experiment was done to examine the binding of Bhtr-5 after pretreatment with htr-1 and htr-9. It was found that htr-9 did not inhibit the binding of Bhtr-5, whereas htr-1 inhibited the Bhtr-5 binding (Fig. 2, C and D). This indicates that the epitope recognized by htr-5 is not identical to that recognized by htr-9. The results in Figs. 1 and 2 are summarized in a qualitative manner in Table I.

Effects of TNF Receptor Antibodies on BrTNF-α Binding to U937 and HL-60 Cells. As htr-5 was found to inhibit the binding of Bhtr-9, it was of interest to see if this antibody also inhibited BrTNF-α binding. U937 and HL-60 cells were pretreated with
TABLE I
Specificity of Binding of TNF Receptor Antibodies to U937 Cells

| Antibody | TNF-α | TNF-β | htr-9 | htr-5 | htr-1 |
|----------|-------|-------|-------|-------|-------|
| htr-9    | Yes   | Yes   | —     | Yes   | Yes   |
| htr-5    | Yes   | Minimally | No | —     | Yes   |
| htr-1†   | Yes   | Minimally | Yes | No | —     |

* Binding was determined by flow cytometry and the table is a qualitative summary of the results in Figs. 1 and 2.
† Data not shown.

fTNF-α or htr-5 and thereafter stained with BrTNF-α and analyzed by flow cytometry. As shown in Fig. 3 A htr-5 only minimally inhibited the binding of BrTNF-α to U937 cells. However, htr-5 was far more effective to inhibit the binding of BrTNF-α to HL-60 cells (Fig. 3 B). Similar results were also obtained by using htr-9 pretreatment (data not shown).

**Biological Effects of TNF Receptor Antibodies on U937 Cells.** The htr-1, htr-5, and htr-9 antibodies bind to epitopes associated with a TNF receptor (Fig. 1). Efforts...
were therefore made to determine whether these antibodies could mimic or block the biological effects of TNF-α in different target cell systems. The htr-1, htr-5, and htr-9 antibodies were first tested if they had any cytotoxic activity on U937 cells. The results shown in Fig. 4 demonstrate that htr-1 and htr-9 induced a high degree of cytolysis, whereas htr-5 was ineffective in this respect. When compared in molar terms, $\sim 10^3$ to $10^4$ times more of htr-9 than rTNF-α was needed in order to mediate a comparable level of cytolytic activity. The IgM antibody, htr-1, was $\sim 100$ times more effective than htr-9 in inducing cytotoxicity of U937 (Fig. 4). These data suggest that htr-1 and htr-9 recognize an epitope of a TNF receptor that is involved in inducing the cytotoxic activity of TNF-α. As the binding studies indicated that htr-5 inhibited htr-9 binding, it was of interest to examine whether htr-5 could also inhibit the cytotoxic activity of htr-9. Cytotoxicity mediated by htr-9 was subsequently determined in the absence and presence of 10 μg/ml htr-5. As expected, htr-5 completely inhibited the cytotoxic activity of htr-9 (Fig. 4 B). It was also tested if htr-5 was able to inhibit the cytotoxic action of rTNF-α. The results in Fig. 5 revealed

![Figure 4](image-url)

**Figure 4.** (A) Cytotoxic effects of TNF receptor antibodies (htr-1, htr-5, and htr-9) on U937 cells. The bioactivity of htr-1 was diluted out between 0.01 and 0.1 ng/ml (data not shown). (B) Inhibition of htr-9 induced cytotoxicity on U937 cells with htr-5 antibodies. Cytotoxicity was determined in a 20-h ³¹Cr-release assay in the absence or presence of 10 μg/ml htr-5 (+ htr-5).
that htr-5 did not completely inhibit the cytotoxicity, but shifted the rTNF-α dose-response curve ~10-fold. The control antibody pfa-11 did not inhibit htr-9- or TNF-α-mediated cytolysis (data not shown).

Biological Effects of TNF Receptor Antibodies on Fs4 Fibroblasts. The different biological effects of TNF-α depend on the target cells used. It was therefore of interest to see if the TNF receptor antibodies also could mimic the growth-stimulating activity of TNF-α on human Fs4 fibroblasts. Both htr-1 and htr-9 induced a dose related increase in thymidine incorporation in Fs4 cells (Fig. 6 A). In contrast, htr-5 did not have any effect on thymidine incorporation (Fig. 6 A). Also in this system, htr-1 was the most effective antibody in mimicking TNF-α activity (Fig. 6 A). Approximately 10² times more of htr-9 than rTNF-α was needed in order to obtain comparable proliferative activities (data not shown). As was the case with U937, htr-5 was able to completely block the proliferative activity induced by htr-9 on Fs4 cells (Fig. 6 B). In contrast, rTNF-α induced proliferation of Fs4 cells was not affected by htr-5 treatment (data not shown). These data suggest that the epitope recognized by htr-9 antibody is involved in TNF-α signal for stimulating the growth of Fs4 cells.

Biological Effects of TNF Receptor Antibodies on Human Endothelial Cells. The effects of TNF receptor antibodies on IL-6 production by HEC were studied in a direct comparison with the effects of rTNF-α and the results are presented in Table II. Consistent with data reported recently (18) rTNF-α caused a substantial stimulation of IL-6 production after 24 h of incubation. While pfa-11 and htr-5 had little effects, antibody htr-9 induced a marked increase in IL-6 production reaching 2.45 folds greater than the amounts detected in control HEC cultures (Table II). The presence of htr-1 also caused a marked enhancement of IL-6 production (not shown).

One of the important biological properties of TNF-α is its ability to increase the adhesiveness of HEC (19). Therefore, the ability of htr-9 antibody to mimic TNF-α effect was tested in an adhesion assay using ⁵¹Cr HL-60 and the results are
Figures 6. (A) Effects of htr-1, htr-5, and htr-9 antibodies on proliferation of Fs4 cells. The antibodies were added to Fs4 cells for 72 h before incorporation of radioactive thymidine was determined. (B) Inhibition of htr-9-induced increase in proliferation of Fs4 cells by htr-5 antibodies. Thymidine incorporation was estimated in the absence or in presence of 10 μg/ml htr-5 (+htr-5).

Discussion
In this study antibodies against a TNF receptor (htr-1, htr-5, htr-9) have been used to study their binding pattern to U937 cells and their biological effects on U937 cells, Fs4 fibroblasts, and endothelial cells. The htr-1, htr-5, and htr-9 antibodies presented in Table III. HEC treated with htr-9 (0.1-1 μg/ml) showed an increased adhesiveness similar to that observed after treatment with rTNF-α as reflected by the adhesion of 51Cr HL-60 cells. Htr-5 did not have any effect on the adhesiveness. However, htr-5 was able to completely block the effect of htr-9 also in this system. Furthermore, htr-5 could partially inhibit rTNF-α-induced adhesiveness. The treatment of HEC monolayers with an irrelevant antibody, pfa-11, did not have any effect (data not shown), indicating the specificity of the observed htr-9 effect. The results presented in Tables II and III were confirmed in two additional experiments.
The Influence of Antibodies to TNF Receptor on IL-6 Production by HEC

Cultures of confluent HEC monolayers were incubated in duplicate with and without the addition of various agents as indicated. After 24 h of incubation the supernatants were collected and tested for IL-6 activity. The data are presented as the mean of triplicate IL-6 determination. SD <10%. Stimulation index is calculated by dividing IL-6 values from test cultures by IL-6 values in control cultures.

Table II

| Agent added to HEC cultures | IL-6 | Stimulation* index |
|-----------------------------|------|-------------------|
|                            | pg/ml|                   |
| None                       | 180  | 1.0               |
| rTNF-α 10 U/ml             | 509  | 2.83              |
| rTNF-α 100 U/ml            | 801  | 4.45              |
| Htr-9 0.25 μg/ml           | 342  | 1.90              |
| Htr-9 2.50 μg/ml           | 452  | 2.40              |
| Htr-5 0.25 μg/ml           | 287  | 1.59              |
| Htr-5 2.50 μg/ml           | 227  | 1.26              |
| Htr-11 2.5 μg/ml           | 220  | 1.22              |

* Cultures of confluent HEC monolayers were incubated in duplicate with and without the addition of various agents as indicated. After 24 h of incubation the supernatants were collected and tested for IL-6 activity. The data are presented as the mean of triplicate IL-6 determination. SD <10%. Stimulation index is calculated by dividing IL-6 values from test cultures by IL-6 values in control cultures.

Adherence of HL-60 to Human Endothelial Cells (HEC) after Treatment with Antibodies to a TNF Receptor

| Treatment of HEC cultures | Percent adherence of HL-60 |
|---------------------------|---------------------------|
| None                      | 4                         |
| rTNF-α (10 U/ml)          | 96                        |
| Htr-9 (0.1 μg/ml)         | 47                        |
| Htr-9 (1 μg/ml)           | 73                        |
| Htr-5 (1 μg/ml)           | 5                         |
| Htr-9 (0.1 μg/ml) + htr-5 (1 μg/ml) | 6                      |
| Htr-9 (1 μg/ml) + htr-5 (1 μg/ml) | 6                      |
| rTNF-α (10 U/ml) + htr-5 (1 μg/ml) | 69                      |

Cultures of confluent HEC monolayers were incubated in medium only and in medium plus either rTNF-α, htr-9, or htr-5 as indicated. After 4 h, the cultures were washed twice with HBSS and each culture received 10^5 ^{51}Cr HL-60 in 0.25 ml medium and was further incubated for 1 h. Nonadherent cells were aspirated, the cultures were washed, and adherent ^{51}Cr HL-60 were lyed for determination of retained radioactivity. Each treatment was performed in duplicate and the data are presented as mean cpm from four lysate aliquots counted.
bodies and flow cytometric quantification. htr-5 and htr-9 both bound to the plasma membrane of U937 cells. Inhibition of the binding by pretreatment with rTNF-α demonstrated the specificity of these antibodies for the TNF receptor. Furthermore, the binding studies demonstrated that htr-9 recognized an epitope that was shared by htr-5 and htr-1, while htr-5 recognized an epitope that was shared by htr-1 but not by htr-9. The most likely explanation of these results is that htr-5 and htr-9 recognize two different but spatially related epitopes on the same TNF receptor molecule. It is unclear whether the epitopes recognized by htr-1 and htr-9 are identical. The result that htr-1, but not htr-9, inhibited htr-5 binding could be due to the fact that htr-1 is an IgM antibody that is larger than htr-9 and thereby can cause steric hindrance for htr-5 binding.

To our surprise we found that TNFβ did not reduce the binding of htr-5 to U937 cells to the same extent as TNF-α did. Many of the biological activities of TNF-α are shared by TNF-β (3, 4). However, there are differences between TNF-α and TNF-β activities in some systems. In human endothelial cells a higher dose of TNF-β is needed compared with TNF-α in order to induce similar activities (20). Aggarwal et al. have found that TNF-α and TNF-β compete with 125I-TNF-α for binding to the same receptor (12). The finding that htr-9 binding was blocked by rTNF-α and rTNF-β is consistent with the presence of a common receptor for both forms of TNF. However, the presence of specific epitopes for each cytokine is evident from our binding studies with htr-5. These epitopes may determine the affinity of each cytokine for corresponding receptor-cytokine interaction.

We have demonstrated that the htr-1 and htr-9 antibodies against the TNF receptor mimic the action of rTNF-α on U937 cells, Fs4 fibroblasts, and endothelial cells. The htr-5 antibody had no TNF-α activity in these systems. However, htr-5 completely inhibited the biological activity of htr-9 on U937 cells and on Fs4 fibroblasts. These results further give support to the observation that htr-5 and htr-9 recognize different but overlapping epitopes of the TNF receptor. Furthermore, the data strongly indicate that a common epitope of a TNF receptor is involved in inducing cytotoxicity of tumor cells, proliferation of fibroblasts, and activation of endothelial cells for IL-6 production and tumor cell adherence. The biological activities of htr-1 and htr-9 were different than the recently described antibody (anti-Fas) that was reported to recognize an antigen associated with a TNF receptor and that killed cells that were not susceptible to the cytolitic action of TNF-α (21). Our data suggest that htr-1 and htr-9 detect an epitope of a TNF receptor that is present on TNF-sensitive U937 cells, Fs4 fibroblasts and endothelial cells and induce TNF-α activity. The results showing TNF-like activity for the antibody htr-9 are in accordance with the observation that the same antibody as well as TNF activate the transcription factor NF-κB in the nuclei of HL-60 and HEp2 cells (Hohmann, H., H. Loetscher, M. Brockhaus, R. Remy, and A. P. G. M. van Loon, manuscript submitted for publication).

The binding studies with BrTNF-α revealed that pretreatment with htr-5 or htr-9 only partially reduced BrTNF-α binding to U937 cells, whereas the inhibition of BrTNF-α binding by htr-5 or htr-9 pretreatment was much more pronounced in HL-60 cells. In addition, htr-5 only partially inhibited the biological activity of TNF-α on U937 and endothelial cells but not on Fs4 cells. These results could be due to the far lower affinity of the antibodies for the TNF receptor compared with the ligand.
It is also possible that TNF receptors other than the one recognized by htr-5 and htr-9 are involved in binding and signaling of TNF-α in different cell types. The data also suggest that there are differences between cell types with regard to expression of TNF receptors as HL-60 cells express more of the receptor recognized by htr-5 compared with U937 cells. These results are in accordance with Brockhaus and Loetscher (manuscript submitted for publication) who have identified two types of TNF receptors on human cell lines by using mAbs. The functional relationship between the apparently different TNF receptor types is not clear and requires further studies.

Summary

Three different antibodies against a human TNF receptor (htr-1, htr-5, and htr-9) have been examined for their binding pattern to U937 cells and ability to mimic TNF-α activity in U937 cells, Fs4 fibroblasts, and human endothelial cells. Flow cytometric analysis revealed that htr-5 and htr-9 bound specifically to a TNF receptor on U937 cells that could be blocked by pretreatment with rTNF-α. Pretreatment of U937 cells with rTNF-α blocked the binding of htr-9, but to a lesser extent htr-5 binding. Pretreatment with htr-5 inhibited the binding of htr-9 to U937 cells while pretreatment with htr-9 did not inhibit htr-5 binding. These results indicate that htr-5 and htr-9 recognize distinct but overlapping epitopes of a human TNF receptor on U937 cells and that htr-5 may be close to a TNF-α-specific domain of the binding site. Pretreatment with htr-5 or htr-9 only minimally reduced binding of BrTNF-α to U937 cells; however, these antibodies were much more effective in inhibiting BrTNF-α binding to HL-60 cells. Furthermore, it was found that htr-1 and htr-9, but not htr-5, had TNF-α activity on U937 cells, Fs4 fibroblasts, and endothelial cells and that the TNF-α activity induced by htr-9 was completely inhibited by htr-5. However, the cytotoxic activity of TNF-α was only partially inhibited by htr-5 on U937 cells while htr-5 had no effect on TNF-α activity on Fs4 cells. The data suggest that a common epitope is involved in inducing TNF-α activity in three different cell systems.

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