TNF- α Receptor Knockout Mice Are Protected from the Fibroproliferative Effects of Inhaled Asbestos Fibers

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We have demonstrated that C57BL/6–129 hybrid mice with genes for both the 55kd and 75kd receptors for TNF- α knocked out (TNF- α RKO) fail to develop fibroproliferative lesions after asbestos exposure. There is good evidence that TNF- α plays a major role in mediating interstitial pulmonary fibrosis. Our findings support this view and we present here new data obtained by *in situ* hybridization showing that expression of the genes coding for transforming growth factor α (TGF- α) and platelet-derived growth factor A-chain (PDGF-A) is reduced in the TNF- α RKO mice compared with control animals. In accordance with this observation, data on bromodeoxyuridine (BrdU) incorporation in the lungs of the TNF- α RKO mice show no increases over unexposed control animals. In contrast, wild-type control mice exposed to asbestos exhibit 15- to 20-fold increases in BrdU uptake and consequently develop fibrogenic lesions. Even though the levels of TNF- α gene expression and protein production were increased in the asbestos-exposed TNF- α RKO mice, the lack of receptor signaling protected the mice from developing fibroproliferative lesions. We agree with the view that TNF- α is essential for the development of interstitial pulmonary fibrosis and postulate that TNF- α mediates its effects through activation of other growth factors such as PDGF and TGF- α that control cell growth and matrix production. (*Am J Pathol* 1998, 153:1839–1847)

Fibroproliferative lung disease afflicts millions of individuals worldwide. The resultant scarring causes restrictive lung disease, shortness of breath, and increased morbidity and mortality.¹ The biochemical and molecular mechanisms that mediate the disease process remain undefined, although an expanding body of literature supports the view that an interacting cascade of cytokines and growth factors is essential for the development of

pulmonary fibrosis.² We have focused on a group of these factors that are expressed rapidly (within hours) at the sites of initial lung injury induced by inhaled asbestos, a well-known fibrogenic mineral.^{3–5}

Among the many cytokines and peptide growth factors found in human and animal lungs with fibrogenic disease are platelet-derived growth factor (PDGF),⁶ transforming growth factors α ⁷ and β ⁸ (TGF- α and TGF- β), and tumor necrosis factor α (TNF- α).⁹ PDGF is the most potent mesenchymal cell mitogen yet described⁶ and TGF- α is a powerful inducer of epithelial and mesenchymal cell proliferation.⁷ On the other hand, TGF- β generally blocks cell growth but is a potent stimulus for extracellular matrix production.⁸ TNF- α has been postulated as a central mediator of fibrogenic lung disease caused by such diverse agents as bleomycin and silica.¹⁰ TNF- α clearly is a multipotent cytokine, acting on the one hand as a growth factor and on the other as an activator of gene expression.^{11,12} To determine the role TNF- α might play in the initial fibroproliferative response to lung injury, we have exposed mice to fibrogenic asbestos fibers for a single 5-hour time period. This brief exposure induces a fibroproliferative disease process localized initially at bronchiolar-alveolar duct regions of the lung.^{4,5,13} Here we show that mice deficient in both the 55kd and 75kd receptors for TNF- α are protected from the initial fibroproliferative effects of inhaled asbestos fibers. We also demonstrate that although levels of TNF- α expression increased in these animals, expression of PDGF and TGF- α are significantly reduced in the receptor knockout mice, supporting the view that TNF- α may exert its effects on disease development by controlling growth factor synthesis.

Materials and Methods

Mice

Mice with mutations in both the p55 and p75 TNF receptor genes have been described previously.¹⁴ These mice

Supported by National Institutes of Health Grants RO1ES60766 and RO1HL60532, the Tulane/Xavier Center for Bioenvironmental Research, and the Tulane Cancer Center.

Accepted for publication September 12, 1998.

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(kindly supplied by Dr. Jacques Peschon, Immunex Corporation, Seattle) were generated by disrupting the individual receptor genes and then interbreeding the single-receptor knockout lines. TNFR double-knockout mice were maintained on a mixed genetic background of the C57BL/6 and 129 inbred strains (B6129). B6129 F2 hybrid mice and C57BL/6 mice purchased from the Jackson Laboratories (Bar Harbor, ME) were used as wild-type controls. All mice were housed according to NIH guidelines under specific pathogen-free conditions.

Asbestos Exposure and Tissue Preparation

Mice were exposed to asbestos in a 39-L inner aluminum chamber containing the exposure atmosphere within a 1.5-m³ stainless steel Rochester outer chamber. Asbestos aerosol was generated from California chrysotile¹⁵ and passed through a vertical elutriator to allow only particles <10 μ m aerodynamic equivalent diameter to enter the chamber. Mice were exposed via the nose only. Dust concentrations in the exposure chamber were measured by sampling onto 37-mm PVC membrane filters placed in unused animal ports followed by gravimetric analysis of the samples. TNFR double knockout mice (p55^{-/-} p75^{-/-}) were exposed to an aerosol of chrysotile asbestos (10 mg/m³ respirable mass) or to room air (sham) for 5 hours. C57BL/6 and B6129 F2 hybrid mice (Jackson Laboratories) were exposed simultaneously as background controls. Five animals per group were euthanized at periods of 0 hours, 48 hours, and 2 weeks after the single 5-hour exposure. Lungs were perfused through the trachea with 10% neutral buffered formalin at a pressure of 25 cm H₂O for 30 minutes. After perfusion, the trachea was clamped and the lungs were removed from the chest cavity and placed in fresh fixative for 16 hours at 4°C. After fixation, lungs were embedded in paraffin, and 5- μ m-thick sections were cut onto positively charged slides for immunohistochemistry and *in situ* hybridization. The general histopathological appearance of tissues was assessed after routine hematoxylin and eosin staining. Before starting any exposures, five animals were sacrificed and the fixed lungs were processed for routine histopathology to be sure that the mice were healthy. The exposure and tissue preparation protocols were carried out two separate times several months apart with no apparent differences in any of the parameters studied (see Results).

In Situ Hybridization

Tissue and Probe Preparation

Tissue sections for *in situ* hybridization were kept at 4°C until used. The nonradioactive *in situ* hybridization method used in this experiment has been described previously.^{4,5} The cDNAs encoding rat PDGF-A, rat TGF- α , and mouse TNF- α (kindly provided by Dr. Dai Katayose, NHLBI/NIH, Bethesda, MD; Dr. David Lee, University of North Carolina at Chapel Hill; and Dr. Bruce Beutler, University of Texas Southwestern Medical Cen-

ter, respectively) were used as templates to generate RNA probes. Labeled cRNA probes for PDGF-A, TGF- α , and TNF- α were transcribed from plasmids containing restriction fragments of growth factor cDNAs as follows: PDGF-A, a 0.8 kb *Sma*I fragment in pBluescript KS+;⁵ TGF- α , a 2.0 kb *Eco*RI/*Sa*I fragment in pGEM4;⁴ TNF- α , a 1.1 kb *Pst*I/*Eco*RI fragment in pGEM3.¹⁶ Linearized plasmids were used as templates for *in vitro* transcription reactions to produce digoxigenin-11-UTP-labeled anti-sense and sense riboprobes with T7 and T3 RNA polymerase (Genius 4 RNA labeling Kit, Boehringer Mannheim, Indianapolis, IN).

Hybridization

Hybridization of cRNA probes to lung tissue sections was performed as described previously.^{4,5} Slides were counterstained with Mayer's hematoxylin.

Immunohistochemistry

TNF- α

Immunohistochemical staining for TNF- α was performed using the immunoperoxidase technique described previously.^{4,5} Briefly, slides were incubated in methanol containing 0.3% hydrogen peroxide for 30 minutes and then in 5% normal goat serum for 30 minutes. Slides were incubated with a rabbit anti-mouse TNF- α antibody (1:100, a kind gift from Dr. Steven Kunkel, University of Michigan, Ann Arbor, MI) at room temperature for 1 hour. A parallel set of sections was incubated with the same dilution of normal rabbit serum as a control for nonspecific binding. The slides were then incubated with biotinylated goat anti-rabbit (1:4,000, Jackson Immunoresearch, West Grove, PA) and streptavidin-horseradish peroxidase (1:2000, Jackson Immunoresearch). Peroxidase activity was visualized with a 10-minute incubation in 0.05 mol/L Tris-HCl, pH 7.6, containing 200 μ g/ml diaminobenzidine and 0.006% hydrogen peroxide. The slides were counterstained with Lerner-3 hematoxylin (Lerner, Pittsburgh, PA).

Bromodeoxyuridine (BrdU) Labeling

The asbestos-exposed and control mice were injected intraperitoneally with BrdU (50 mg/kg) 4 hours before sacrifice as reported previously.¹⁷ Sections were pre-treated with 0.01% trypsin in 0.05 mol/L Tris-HCl, pH 7.8, containing 0.1% CaCl₂ for 6–10 minutes at 37°C. Sections were incubated in methanol containing 0.3% hydrogen peroxide for 30 minutes and then in 5% normal goat serum for 30 minutes. The slides were incubated with a mouse monoclonal antibody against BrdU (clone B44, 1:100, Becton Dickinson, San Jose, CA) at room temperature for 1 hour. A parallel set of sections was incubated with the same dilution of normal rabbit serum as a control for nonspecific binding. Following biotin-conjugated goat anti-mouse (1:4000) and streptavidin-horseradish peroxidase (1:2000) incubation, peroxidase activity was visu-

Figure 1. Histopathology. **A:** Normal (B6129) mouse lung from an air-exposed control animal. The arrow indicates the alveolar duct bifurcation region that exhibits a rapid fibroproliferative response after asbestos exposure (compare with **B**). **B:** Enlarged bifurcation (**box**) in a B6129 mouse 48 hrs after exposure to chrysotile asbestos. The lesion is hypercellular and hypertrophic, with increased connective tissue matrix, macrophages, and interstitial cells^{13,18} (inset, $\times 40$). **C:** A bronchiolar-alveolar duct lesion (**arrow**) in a B6129 mouse 48 hours after exposure. **D:** The TNF- α RKO mice fail to develop the fibroproliferative lesions consequent to asbestos exposure. This knockout animal exhibits a normal duct bifurcation (**arrow**) 48 hours after asbestos exposure. Bars, 20 microns.

alized with diaminobenzidine as described above. The slides were counterstained with Lerner-3 hematoxylin (Lerner).

Quantitative Analysis of BrdU Labeling

BrdU labeling was quantitated by counting labeled cells at bronchiolar-alveolar duct junctions. Two histological sections per lung were prepared and analyzed from 5 different animals at each of 3 time points (0 hours, 48 hours, and 2 weeks) after a single 5-hour asbestos or sham exposure. Bronchiolar/alveolar anatomical units were selected at random from each animal for analysis. Each anatomical unit consisted of the following features: a terminal bronchiole, alveolar duct walls between the terminal bronchiole and first alveolar duct bifurcation, and a first alveolar duct bifurcation. A total of 1500 cells, typically comprising 4–6 anatomical units, were counted

per animal. A BrdU labeling index was calculated by dividing the number of BrdU-positive nuclei by the total number of cells counted in the given units. Differences between groups were analyzed by one-way analysis of variance.

Results

Histopathology

The TNF- α receptor knockout (TNF- α RKO) mice and wild-type mice of the same genetic background, ie, C57BL/6–129 F₂ hybrids (B6129), were exposed simultaneously to an aerosol of chrysotile asbestos fibers. Additional groups of these mice were exposed to room air as negative controls. We have shown previously that 5 hours of exposure to chrysotile asbestos fibers induces

Figure 2. Bromodeoxyuridine Incorporation. **A:** Bromodeoxyuridine (BrdU) is used as a measure of cell proliferation. The large number of heavily stained cells in the mouse intestine serves as a positive control for BrdU incorporation in the lungs. **B:** The bronchiolar-alveolar regions of normal B6129 air-exposed mice exhibited occasional (~1–2%) labeled cells (arrowhead). **C:** By 48 hours after exposure in B6129 hybrids, the terminal bronchiolar walls and duct bifurcations (box) contained numerous BrdU-labeled epithelial (arrowheads) and interstitial (arrow) cells. The increases approached 15- to 20-fold (see Figure 3) in the C57 and B6129 mice exposed to asbestos and remained significantly elevated for at least 2 weeks postexposure (see Figure 3). The boxed bifurcation and a bronchiolar wall are enlarged in the insets (magnification, $\times 40$). **D:** Cells of the bronchiolar-alveolar duct regions in B6129 knockout mice failed to incorporate BrdU at levels above background. Bars, 20 microns.

the development of fibroproliferative lesions at bronchiolar-alveolar duct (BAD) junctions in rats and mice.^{3–5,13} C57BL/6 (C57) mice exposed to asbestos at the same time served as positive controls to contrast the response of the B6129 hybrids and the TNF- α RKO mice exposed identically. Figure 1 shows typical histopathological sections from these animals. The air-exposed B6129 mice exhibited normal architecture with no inflammatory lesions in any animals (Figure 1A). The asbestos-exposed C57 and B6129 mice developed typical lesions at the BAD junctions 48 hours after exposure (Figure 1, B and C). These lesions have been described in detail previously^{4,5,13} and are hypercellular and hypertrophic, with numerous alveolar and interstitial macrophages as well as asbestos fibers and increased numbers of mesenchymal cells.¹⁸ In contrast, the TNF- α RKO mice failed to develop significant lesions (Figure 1D). An experienced histopathologist, blinded as to the identity of the tissue sections from the groups of animals, placed the great

majority of the asbestos-exposed TNF- α RKO mice in the normal category. A few of the animals had increased alveolar macrophages at the BAD junctions and could be identified as asbestos-exposed, but there were no fibroproliferative lesions in these animals.

BrdU Incorporation

BrdU incorporation is a valuable measure of cell proliferation.¹⁷ As expected, the air-exposed mice had few stained cells at any time after exposure (Figure 2B). Also as expected, the C57 and B6129 mice exhibited numerous densely labeled cells in the developing lesions. There was no staining immediately after exposure, but at 48 hours after exposure, numerous interstitial, epithelial, and bronchiolar Clara cells had incorporated BrdU (Figure 2C). Analysis of the percentages of labeled cells demonstrated that the increased staining persisted for at least 2 weeks after exposure (Figure 3).

Figure 3. Analysis of BrdU incorporation. Analysis of the percentages of cells incorporating BrdU in the four groups of mice studied. By 48 hours after exposure to asbestos and persisting for at least 2 weeks, the cells in C57 and B6129 hybrid mice exhibited significant increases over air-exposed controls and TNF- α RKO animals exposed to asbestos. These knockout mice remained at control levels throughout the experiment despite being exposed to the same dose of asbestos that caused fibroproliferative lesions in the background controls. **a**, $P < 0.01$; **b**, $P < 0.05$ vs. the air-exposed controls and asbestos-exposed TNF- α RKO mice.

The TNF- α RKO mice exhibited very few BrdU-stained cells at any time after exposure (Figure 2D and Figure 3), and these animals had significantly fewer cells incorporating BrdU than the B6129 hybrid controls. There were no significant differences between the percentages of labeled cells in air-exposed B6129 mice and the asbestos-exposed TNF- α RKO animals.

Growth Factor Expression

In situ hybridization was carried out to determine the distribution of TNF- α , PDGF-A, and TGF- α mRNA expression. Figure 4 shows that asbestos-exposed B6129 mice exhibited strong hybridization of the mRNAs for each of the three growth factors studied at 48 hours after exposure. Air-exposed animals were essentially negative. The sense strand of the mRNAs served as negative controls for the *in situ* hybridization technique (see Figure 5A). Most interesting was our finding that expression of the mRNAs for PDGF-A and TGF- α were markedly reduced in asbestos-exposed TNF- α RKO mice compared to the asbestos-exposed wild-type mice. In contrast, dense hybridization of the TNF- α mRNA was observed in both these animal groups after asbestos exposure (Figure 4, G and H). Immunohistochemical staining of TNF- α protein in sections from the TNF- α RKO (Figure 5D) and B6129 mice (Figure 5C) confirmed that asbestos exposure induces TNF- α expression regardless of whether fibroproliferative lesions are developing. TNF- α gene and protein expression were observed primarily in bronchiolar-alveolar epithelial cells and alveolar macrophages (Figures 4 and 5).

Discussion

We have demonstrated that mice lacking the genes for both the 55kd and 75kd membrane receptors for TNF- α

fail to develop fibroproliferative lung lesions following brief exposure to chrysotile asbestos fibers. The lesions developed as expected at the bronchiolar-alveolar duct (BAD) junctions of asbestos-exposed C57 and wild-type mice of the same genetic background as the TNF- α RKO knockout animals. These wild-type control animals exhibited dense staining of BrdU in multiple cell types of the developing lesions, and there was strong expression of the mRNAs coding for TNF- α , TGF- α , and PDGF-A. These findings are consistent with the current postulate that fibrogenic lung disease develops as a result of growth factor-induced cell proliferation. These data also support the view that TNF- α plays a major role in mediating the fibroproliferative process. Thus, in mice with normal TNF- α receptors there is expression of growth factors, such as PDGF-A and TGF- α , that can induce mesenchymal and epithelial cell proliferation respectively. When the TNF- α receptors are lacking, TNF- α gene and protein expression remain up-regulated after asbestos exposure, but PDGF-A and TGF- α are clearly reduced. This could explain the lack of fibroproliferative lesions in the knockout mice.

What is the role of TNF- α as an essential factor in the development of fibroproliferative lung disease? Unfortunately, it is not possible to answer this central question definitively at this time, but the data are consistent with a number of other model systems in which TNF- α appears to play a significant role in several disease processes.

TNF- α was discovered in 1975 as a soluble polypeptide of about 17kd in monomeric form.¹⁹ As a trimeric complex, TNF- α binds to the two demonstrated membrane receptors of 55kd and 75kd. The biology of these receptors is not completely understood, but it appears that both are necessary for TNF- α to produce its maximum effect.²⁰ TNF- α is known to have multiple biological actions on a number of cell types.^{21–23} For example, there is a broad literature on its role in cytolysis and infection.^{20,23} We will confine our discussion to the effects of TNF- α that are most relevant to the fibroproliferative response. Indeed, TNF- α has been implicated as a central mediator in pulmonary fibrogenesis caused by bleomycin,¹⁰ silica,²⁴ and asbestos.²⁵ In addition, TNF- α has been demonstrated in the formation of the collagen associated with chronic arthritis in a mouse model.²⁶ These claims have been made because the processes have been blocked or ameliorated by treatment with anti-TNF- α antibodies (Ab) and/or with recombinant soluble TNF- α receptor (TNF- α -R). In each case cited above, the Abs or the soluble receptor were administered intraperitoneally or intravenously, and they significantly reduced lung collagen accumulation and severity of disease in general.^{10,24,26,27} In one very interesting model, the "moth-eaten" mutant mouse spontaneously develops progressive pulmonary inflammation and fibrosis.²⁸ These animals were found to have high circulating levels of TNF- α and treating them with an anti-TNF- α Ab prevented much of the inflammation and consequent pulmonary fibrosis.²⁸ In addition, Sendai Virus-induced bronchiolar fibrogenesis was inhibited by an antibody to the 55kd TNF- α receptor.²⁹

Figure 4. A: *In situ* hybridization (ISH) of the gene coding for TGF- α in an asbestos-exposed B6129 mouse. Multiple cells are hybridized 48 hours after exposure, including macrophages (arrows) and epithelial cells (arrowheads). TGF- α typically is expressed by few interstitial cells after asbestos exposure (see Reference 4). B: ISH for TGF- α in TNF- α RKO mice resulted in little staining (arrowhead) in the lung 48 hours after asbestos exposure. C: ISH for PDGF-A after asbestos exposure. The alveolar duct lesion (arrow) exhibits staining of multiple epithelial (arrowheads) and interstitial (short arrow) cells 48 hours after exposure in hybrid mice. A detailed study of PDGF gene expression in rats shows a similar pattern (see Reference 5). D: The TNF- α RKO mice not only lack the developing lesions at duct bifurcations (arrow) but also fail to express the gene for PDGF-A except in occasional cells (arrowhead). E: The gene for PDGF-A is demonstrated in hybrid mice at higher magnification in macrophages (arrowhead) and epithelial cells (arrows). F: ISH for TNF- α is clear in the developing lesions (arrow) of hybrid mice as well as in macrophages (M) and epithelial cells (arrowhead). G: Additional ISH of TNF- α in B6129 mice for comparison with the TNF- α RKO animals (see H). H: Despite the lack of developing alveolar duct lesions (arrow), the TNF- α RKO mice exhibit clear ISH for TNF- α (arrowheads). Multiple positive epithelial cells are illustrated at higher magnification in the inset. Bars, 20 microns.

Figure 5. Immunohistochemistry of TNF- α . **A:** Example of an mRNA sense control for ISH in an asbestos-exposed hybrid mouse. This control demonstrates the specificity of the antisense probe and its color reaction product. **B:** IgG control for immunohistochemistry shows very weak background staining in tissue from an asbestos-exposed hybrid mouse. The lesions (arrow) and surrounding macrophages failed to stain. **C:** An anti-TNF- α Ab stains developing alveolar duct lesions (arrowheads) and macrophages (arrows) 48 hours after asbestos exposure in hybrid mice. **D:** The TNF- α RKO mice also exhibit clear staining with the anti-TNF- α Ab in epithelial cells (arrowheads) and macrophages (arrows). Bars, 20 microns.

Thus, there is good evidence that TNF- α holds a strong position on the growing list of cytokines that appear to be essential in mediating fibroproliferative processes. Inasmuch as we have shown that brief inhalation of chrysotile asbestos fibers in rats and mice causes macrophage accumulation, cell injury and proliferation, and fibrogenic lesions,^{4,5,13,18} this model can be used to attempt to understand how TNF- α exerts its multiple effects on these processes. Knockout mice deficient in both the p55 and p75 TNF- α receptors offer several clues because we have been able to make three relevant observations about the exposed mice: (1) none of the knockout animals exhibited enhanced cell proliferation or developed fibrogenic lesions; (2) the levels of TNF- α gene expression and protein production were increased; and (3) the levels of PDGF-A and TGF- α gene expression were reduced. Considered together, these findings demonstrate that TNF- α signaling is an essential event in the development of asbestos-induced fibroproliferative disease. This is in agreement with the findings of a number of other investigations referenced above, implicating TNF- α as a central mediator of lung fibrogenesis in general. In addition,

we suggest that TNF- α exerts its effects on the fibroproliferative process by influencing the expression of other, perhaps more downstream factors, like PDGF and TGF- α , that bind to their own cell surface receptors. PDGF-A and -B are the most potent mesenchymal cell mitogens yet described,⁶ while TGF- α is a powerful epithelial cell mitogen.⁷ Although our data suggest that TNF- α receptor signaling is essential for the development of fibroproliferative lesions, further experiments will be necessary to establish whether or not TNF- α expression is necessary for the elaboration of other key growth factors. It is clear that TNF- α has a direct influence on the expression of factors such as TGF- β ,^{30,31} and we have new, as yet unpublished data showing that TGF- β 1 expression is also reduced in the TNF- α RKO mice. In addition, TNF- α mediates many of its effects through the transcription factor NF- κ B,³² suggesting the possibility of activating other cytokines that are regulated by this factor.³²

We have focused here on the relationship between TNF- α and growth factors, but there are other scenarios in which TNF- α could influence fibrogenic disease.

Briefly, it has become apparent that increased TNF- α induces expression of collagenase³³ but disrupts the normal attachment of fibroblasts to their extracellular matrix.^{34,35} This reportedly is due to down-regulation of collagen-specific receptors, resulting in decreased turnover of extracellular matrix. Finally, TNF- α has been shown to enhance the release of superoxide ions³⁶ and it is clear that cellular injury from such anions can lead to fibrogenic disease.³⁷ Clearly, TNF- α has multiple influences on a wide variety of inflammatory events³⁸ that are beyond the scope of the experiments presented here.

In summary, we have shown that mice lacking receptors for both the 55kd and 75kd receptors for TNF- α are protected from the fibrogenic effects of inhaled asbestos fibers. We have presented data supporting the postulate that TNF- α is essential for the development of the fibroproliferative process through its effects on the expression of growth factors such as PDGF, TGF- α , and TGF- β that control cell growth and matrix production. Even though the TNF- α mRNA is up-regulated and there is increased protein, the lack of TNF- α receptor signaling protected the mice. Further experiments will be necessary to discover the mechanisms through which TNF- α influences the expression and biological activities of the factors that could more proximally mediate fibroproliferative lung disease.

Acknowledgments

We thank the staff of Tulane Medical Center Anatomical Histopathology Laboratory for technical assistance and Ms. Odette Marquez for preparation of the manuscript.

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