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Chapter 4

IL-10 in the pathogenesis of minimal change nephrotic syndrome
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Minimal change nephrotic syndrome (MCNS) is the most common form of nephrotic syndrome (NS) in children. Clinical and experimental studies have documented an association of MCNS with activation of the immune system toward a T-helper2 phenotype. To establish the involvement of circulating cytokines in the pathogenesis of MCNS, we collected peripheral blood mononuclear cells (PBMC) and plasma from patients with MCNS during relapse (n=16) and remission (n=16), and from a nephrotic control group of 7 patients with NS primarily caused by endogenous alterations within the glomerular filter. RNA was extracted from freshly isolated PBMC and quantitative real-time PCR was performed to study expression of IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, TNF-α, and IFN-γ. Out of these cytokines, only the expression of IL-10 mRNA was significantly and specifically upregulated in relapsing MCNS patients, compared both to MCNS in remission and to nephrotic controls. Correspondingly, IL-10 levels in plasma were increased as well. The expression of IL-13 mRNA was upregulated in both nephrotic patient groups (relapsing MCNS and nephrotic controls) compared to patients in remission. In situ hybridization and Western blotting showed that podocytes express the functional IL-10R1/IL-10R2 receptor complex, which binds IL-10 and signals upon ligation with IL-10. In conclusion, proteinuria in MCNS is associated with an elevated expression of IL-10 by PBMC, which may cause signaling in podocytes. It remains to be established whether IL-10 plays a crucial role in the pathogenesis of MCNS by effects on the glomerular capillary filter.

Idiopathic nephrotic syndrome represents a heterogeneous group of glomerulopathies accounting for 80 to 90% of cases with nephrotic syndrome (NS) in children and for 20 to 30% of cases in adults. Depending on the patient’s response to treatment with corticosteroids, idiopathic NS may be divided into steroid-responsive and steroid-resistant NS. A part of steroid-resistant NS is caused by hereditary mutations in genes encoding structural components of the podocyte foot processes or the slit diaphragm, such as nephrin [1], podocin [2], and α-actinin-4 [3]. The elucidation of the molecular basis of hereditary forms of NS has contributed substantially to our understanding of the pathogenesis of proteinuria [4]. From a clinical point of view, non-hereditary cases are far more common.

The group of non-hereditary idiopathic NS can MCNS produce more IL-13 protein [6] and contain more IL-13 mRNA, as quantified by competitive RT-PCR [7]. Using a subtractive cDNA library screening technique, Sahali et al. recently found a
downregulation of IL-12 receptor β2 mRNA expression by PBMC in MCNS patients during relapse [8], again pointing to the involvement of T-cells and, more specifically, Th2-mediated immunity.

Recently, new tools have been developed to quantitatively measure mRNA, and real-time PCR is an accurate method to study cytokine mRNA expression by PBMC in a quantitative manner [9]. In our study, real-time PCR was used to determine the expression of various Type1 and Type2 cytokines of interest in patients with MCNS during remission and relapse.

**Methods**

**Patients**

Thirty-one patients were studied in total. Twenty-four patients had steroid-sensitive idiopathic NS, of whom 21 had undergone renal biopsy that showed minimal changes with loss of podocyte foot processes in all cases. The other three patients had recurrent NS that responded well to corticosteroids. Blood was collected from patients during long-term remission (n=15) and/or during relapse (n=15). From 6 patients, blood was collected both during remission and during relapse. A control group consisted of 7 patients with NS, due to causes obviously different from MCNS, *i.e.*, mutations in NPHS1 gene (n=3), mutations in NPHS2 gene (n=2), congenital diffuse mesangial sclerosis (n=1) and chronic glomerulonephritis in an inactive phase (n=1). The local Ethical Committee approved the study and informed consent was obtained from all individuals and/or their parents.

**Collection of PBMC and plasma**

Blood samples were collected in the period from 1999 until 2002. At the time of blood collection, all patients were free of immunosuppressive therapy for at least three weeks. The blood was diluted with an equal volume of RPMI 1640 (Invitrogen, Breda, The Netherlands), containing glutamine (2 mM) and 5% heat-inactivated fetal calf serum (Invitrogen). PBMC were isolated over Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and the plasma-containing upper phase was aliquoted and stored at -20°C. PBMC were washed briefly and the pellet was lysed in TRIZOL reagent (Life Technologies) and stored immediately at -80°C until RNA isolation. PBMC were lysed within 1.5 h after blood collection.

**Preparation of cDNA and PCR standards**

Total RNA was extracted from the TRIZOL samples according to the manufacturer’s instructions. For cDNA synthesis, 125 pmol of two-base anchored 5’-(dT)14-d(A/G/C)-d(A/G/C/T)-3’ primers (Amersham Biosciences, Roosendaal, The Netherlands) were annealed on 2.5 μg of RNA during 10 min at 72°C in a 12.5 μl volume. After adding dNTPs, RNAsin, Reactionbuffer and M-MLV transcriptase (Life Technologies) up to a 25 μl volume, reverse transcription was performed for 1 h at 37°C.

PCR products for IL-1 receptor antagonist (IL-1ra), IL-1β, IL-2, IL-4, IL-9 IL-10, IL-13, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and β-actin were prepared as described previously [9] using primers listed in Table 1. PCR products were inserted into a PCR2.1-TOPO vector (Invitrogen) and the reaction product was used to chemically transform One shot bacteria (Invitrogen), all according to the manufacturer’s instructions. For IL-5, bacteria were transformed with a commercial plasmid containing the IL-5 cDNA (from the American Type Culture Collection, Manassas, VA). Plasmid DNA was isolated from positive clones using Qiaprep miniprep system (Qiagen, Valencia, CA).

**Real-time PCR**

Sequences of amplification primers used for real-time PCR are listed in Table 2. Real-time PCR was performed on a LightCycler Instrument using 1 μl of 5-fold diluted cDNA as template in a 10 μl reaction volume, containing 0.5 μM each of sense and antisense primers (Table 2), 2 mM MgCl2, and 1 μl of FastStart DNA Master SYBR Green I reagent (all from Roche Diagnostics, Almere, The Netherlands). The temperature profile was as follows: an initial denaturation for 360 s at 95°C, followed by 40 amplification cycles, each consisting of denaturation for 15 s at 95°C, subsequently annealing for 5 s at a primer specific temperature, and elongation for 10 s
at 72°C, each of these steps with ramp rates of 20°C/s. SybrGreen fluorescence was measured at the end of each elongation phase. Immediately following the last amplification cycle, a melting curve was recorded as follows: after a denaturation at 95°C and subsequent rehybridisation during 15 s at 65°C, the samples were heated to 95°C with a ramp rate of 0.1°C/s with continuous acquisition of SybrGreen fluorescence. In each PCR experiment, the PCR standard for the amplicon of choice was used as a calibrator, in a dilution curve from $10^7$ to $10^2$ copies/µL. The mRNA levels of β-actin were measured as a reference to correct for variable input [9].

### Table 1. Sequences of amplification primers used to obtain PCR standards.

<table>
<thead>
<tr>
<th>mRNA targets</th>
<th>Oligonucleotides (5'→3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1ra</td>
<td>CCCTCCTTCCTGGTCCATTCC</td>
<td>451</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TTCAAGTCGTCATACCTTTCA</td>
<td>495</td>
</tr>
<tr>
<td>IL-2</td>
<td>TGTCACAATAGTGCACCTACT</td>
<td>518</td>
</tr>
<tr>
<td>IL-4</td>
<td>TAATTGCGTCATGTCATCTTA</td>
<td>503</td>
</tr>
<tr>
<td>IL-9</td>
<td>CTGGAATTGATGATGATGAT</td>
<td>506</td>
</tr>
<tr>
<td>IL-10</td>
<td>TGCTTATGCTCAATGTCAT</td>
<td>511</td>
</tr>
<tr>
<td>IL-13</td>
<td>TGGGAGGTGTCACATGCA</td>
<td>521</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>AAGGTTGACTTCTCAGCAGC</td>
<td>529</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CACTGAGCAGAAGGACCATC</td>
<td>509</td>
</tr>
<tr>
<td>β-actin</td>
<td>CCCCTGGAAGAGGTGACTC</td>
<td>509</td>
</tr>
</tbody>
</table>

### Table 2. Sequences of amplification primers used for quantitative PCR. All primer pairs are intron spanning.

<table>
<thead>
<tr>
<th>mRNA targets</th>
<th>Oligonucleotides (5'→3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1ra</td>
<td>GAAGATGAGTGCCTGTCCATT</td>
<td>80</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AAGCAGGCAGTGCCTCAGG</td>
<td>73</td>
</tr>
<tr>
<td>IL-2</td>
<td>CTCAATGCAGAGTATGCA</td>
<td>74</td>
</tr>
<tr>
<td>IL-4</td>
<td>ACTTGAAGATGATGATGAT</td>
<td>71</td>
</tr>
<tr>
<td>IL-5</td>
<td>GAGCTGAAGATGATGATGAT</td>
<td>91</td>
</tr>
<tr>
<td>IL-9</td>
<td>GTGTGATATTGTGATGATGAT</td>
<td>91</td>
</tr>
<tr>
<td>IL-10</td>
<td>AGAGTTGACTTCTCAATGCA</td>
<td>106</td>
</tr>
<tr>
<td>IL-13</td>
<td>TGAGGATGTGATGATGATGAT</td>
<td>76</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TCATTGATGATGATGATGAT</td>
<td>75</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CCCAGGAGCTTCTCCTACT</td>
<td>84</td>
</tr>
<tr>
<td>β-actin</td>
<td>GGATGAGGAGAGGAGTCA</td>
<td>90</td>
</tr>
</tbody>
</table>
ELISA for IL-10 and IL-13

ELISA for IL-10 and IL-13 were commercially obtained (Pelikine compact kits, CLB, Amsterdam, The Netherlands) and used according to the manufacturer’s instructions. The lower detection limits were 1 pg/mL for IL-10 and 0.5 pg/mL for IL-13.

In situ hybridisation

Message RNA expression of IL-10R1 and IL-10R2 was studied by ISH on paraffin sections of human renal tissue. Control human renal tissue was derived from histologically normal parts of kidneys that had been resected because of renal adenocarcinoma (n = 3) and from a living donor (kidney n = 1). Renal tissue was obtained from patients (n = 27) by needle biopsy for diagnostic purposes. The biopsies were diagnosed as MCNS (n = 15), FSGS (n = 5), and IgA nephropathy (n = 7). Nine MCNS patients received steroids at the time of the biopsy, of whom three patients had achieved remission. The other MCNS patients did not receive immunosuppressants yet. The patients with IgA nephropathy had proteinuria. DIG-labeled riboprobes for ISH were prepared from IL-10R1 and IL-10R2-specific cDNA fragments that were obtained by RT-PCR on RNA extracted from human placenta. In the PCR reaction, the following primers were used to obtain cDNA fragments of 462 and 497 bp respectively:

5' - ATTTAGGTGACACTATAGAAGTGACTCT
GACAGTTGGCA-3' and 5'-TAATACGACTCAGTA
TATAGGGCTTCTTGAAAGACCAGAGA-3' for
IL-10R1 and 5'-ATTAGGTGACACTATAGA
AACCCTGACTTTCCAGCCTCAG-3' and 5'-TAA
TACGACTCACTATAAGGTGGTGCTCACAG
ACA-3' for IL-10R2. The underlined primer regions encompass the SP6- and the T7-promoter element sequences. PCR products were inserted into PCR2.1-TOPO vector (Invitrogen) and One shot bacteria (Invitrogen) were transformed subsequently. Identity of the clones was checked by sequencing using an ABI sequencer (Perkin Elmer Corp., Norwalk, CT) with a dye-terminator cycle-sequencing kit (Perkin Elmer). After EcoRI restriction digestion of purified plasmid, the inserts were isolated, purified by phenol extraction, and used as template in an in vitro transcription reaction using T7 and SP6 polymerase to generate antisense and sense DIG-labeled riboprobes, respectively (Roche Diagnostics). ISH was performed as previously described by Fijnvandraat et al. [10]. Subsequently, immunohistochemistry was performed for collagen type IV to provide a counterstain that does not interfere with the cellularly localized ISH signals. Briefly, endogenous peroxidase activity was inactivated, non-specific protein binding sites were blocked with 10% normal goat serum in PBS, and sections were incubated for 1 h with rabbit anti-collagen type IV (Euro-Diagnostica, Arnhem, The Netherlands), diluted to 1:100 in PBS. After washing, sections were incubated with poly-HRP goat anti-rabbit IgG (Powervision, Immunovision Technologies) for 10 min, followed by washing in PBS. HRP activity was detected using 3,3'-diaminobenzidine and hydrogen peroxide. Sections were dehydrated and mounted with Pertex.

Western blotting for STAT phosphorylation

Phosphorylation of signal transducer and activator of transcription (STAT)1, STAT3 and STAT5 in cultured human podocytes was studied by Western blotting, using antibodies directed against STAT3 phosphorylated at Tyr705, STAT5 phosphorylated at Tyr694 (both from Cell Signalling Technology, Beverly, MA, USA), and STAT6 phosphorylated at Tyr641 (New England Biolabs, Beverly, MA), and antibodies directed against total STAT1, total STAT3 (both from BD Biosciences, Erembodegem, Belgium) and total STAT6 (Santa Cruz, CA, USA). The human podocyte line 56/10 A1, a T-SV40 immortalized human glomerular visceral epithelial cell line, was a kind gift from J.D. Sraer [11]. Cells were cultured as previously described [12]. Upon serum starvation for 24 h, cells were exposed to IL-10 (10 ng/mL; Peprotech, Heerhugowaard, The Netherlands) in serum-free media and harvested after 10, 20 and 30 min. Proteins in the cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and were blotted onto PVDF membranes (Millipore). Staining with Ponceau S confirmed effective protein transfer. Blocking was carried out with 5% milk powder (Nutricia, Zoetermeer, The Netherlands) in Tris-buffered saline containing 0.1% Tween 20 (Sigma, St. Louis, MO, USA) (TBSTM) for 1 h at room temperature. The nitrocellulose membranes were then incubated overnight at 4°C with rabbit anti-Phospho-STAT antibodies; incubation with PBS was used as a negative control. After extensive washing with
TBSTM, the membranes were incubated with HRP-conjugated swine-anti rabbit IgG (Dako, Glostrup, Denmark) for 1 h. Binding was visualized using a chemiluminescence detection system (ECL, Amersham, Buckinghamshire, United Kingdom). Subsequently, primary and secondary antibodies were removed by incubation of the membranes in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50°C for 30 min. After washing and blocking in TBSTM for 1 h at room temperature, membranes were reprobed with mouse anti-total-STAT antibodies. Immunodetection was performed as described above, except that HRP-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL, USA) was used as the secondary antibody.

Statistical analysis
Kruskal-Wallis tests and Mann-Whitney tests were applied to evaluate differences between the three patient groups. Paired samples t-test was applied to evaluate differences in the six MCNS patients from whom paired data were obtained. Differences were considered significant when $P < 0.05$.

**Results**

**IL-10 is specifically overexpressed in MCNS**
As shown in Figure 1A, the expression of IL-10 mRNA by PBMC was significantly increased in MCNS patients during relapse, compared both to MCNS in remission ($P < 0.01$) and to the NS control group ($P < 0.05$). The expression of IL-13 mRNA was increased both in nephrotic MCNS patients and in the NS control group, compared to MCNS in remission ($P < 0.05$ for both groups, Figure 1B). There were no differences between the groups in the expression of the other cytokines studied.

ELISA of plasma detected increased expression of IL-10 also at the protein level, showing elevated concentrations of circulating IL-10 during relapse ($P < 0.05$, Figure 1C). IL-13 protein was not detectable in plasma of any of the groups.

We had the opportunity to collect PBMC and plasma of 6 individual MCNS patients both during relapse and in remission. The expression of IL-10 mRNA by PBMC was significantly upregulated during relapse in all patients (Figure 2A), accompanied by elevated IL-10 protein levels in plasma in 4 patients (Figure 2B).

![Figure 1](image.png)

**Figure 1**
A and B. mRNA expression of IL-10 (A) and IL-13 (B) by PBMC from MCNS patients during relapse ($n = 15$) and in remission ($n = 15$), and from a control group of patients with NS primarily caused by endogenous alterations within the glomerular filter ($n = 7$), as detected by quantitative real-time PCR. Values are expressed relative to the mRNA levels of the house-keeping gene β-actin, in arbitrary units. C. Plasma IL-10 levels in the same patients, as detected by ELISA. Horizontal lines denote median values.
IL-10 and minimal change nephrotic syndrome

Figure 2
A. Paired data on mRNA expression of IL-10 by PBMC from MCNS patients (n = 6) during relapse and in remission. Values are expressed relative to the mRNA levels of the house-keeping gene β-actin, in arbitrary units. B. Paired data on the plasma IL-10 protein levels in MCNS patients (n = 6) during relapse and in remission.

A functional IL-10R complex is expressed by podocytes
IL-10 exerts its effects on epithelial and immune cells by binding to a heterodimeric cell surface receptor, composed of IL-10R1 and IL-10R2 chains [13]. Signaling through the heterodimeric receptor involves activation of Janus kinases and tyrosine phosphorylation of STAT1 and STAT3, and in non-macrophage cells STAT5 [14] [15]. By RT-PCR, we found expression for both IL-10R1 and IL-10R2 by cultured human podocytes (data not shown). ISH on human kidney biopsies confirmed mRNA expression of both chains by podocytes in vivo (Figure 3). Expression of the receptor chains by podocytes was scattered and was present in normal kidney and in biopsies diagnosed as MCNS, FSGS, and IgA nephropathy. Focal expression of both chains was observed in endothelial cells and tubular epithelial cells. There were no consistent differences in glomerular expression of the IL-10 receptors between the patient groups.

We next examined phosphorylation of STAT3 and STAT5 in cultured human podocytes upon incubation with IL-10. IL-10 enhanced phosphorylation of both STAT3 and STAT5 in cultured human podocytes within 20 min of incubation (Figure 4), indicating the presence of a functional IL-10R1/2 complex on podocytes in vitro. As expected, phosphorylation of STAT6 was not altered (not shown).

Discussion
This study was undertaken to establish the role of leukocyte-derived cytokines in the pathogenesis of MCNS. The hypothesis that leukocyte-derived cytokines may underlie the induction of MCNS is historically based on clinical observations. Firstly, MCNS is frequently associated with infections and immune-mediated events, such as vaccinations and allergic reactions [16-20] and with lymphoproliferative disease, in particular Hodgkin’s disease [21] and thymoma [22]. Secondly, immunosuppressive therapy is usually effective in inducing complete or partial remission. However, it should be considered that immunosuppressive drugs may also affect non-immune cells, including the podocyte [23]. Nevertheless, experimental studies also pointed to a possible role of leukocytes and cytokines in the pathogenesis of MCNS. CD8-positive T-cells were shown to be clonally expanded during MCNS [24]. Kimata et al found that PBMC

Figure 3. Renal expression of IL-10R1 (A) and IL-10R2 (B) mRNA in a renal biopsy from a patient with MCNS, as detected by ISH. The signal of the DIG-labelled antisense riboprobes is depicted in blue; double-staining for collagen type IV in brown. Control experiments with sense riboprobes showed no specific staining (not shown).
from patients with MCNS produce predominantly the Type2 cytokine IL-13 upon in vitro stimulation [6]. This was confirmed by Yap et al., using semi-quantitative competitive RT-PCR on PBMC directly isolated from the blood, thereby preventing artificial activation of PBMC [7]. Sahali et al. reported on high levels of nuclear factor-κB DNA binding activity in MCNS patients during relapse [25], differential expression of transcripts involved in the T cell receptor mediated complex signaling cascade and decreased expression of IL-12Rβ2 mRNA [8].

Any change in the activation of leukocytes during NS may reflect an immunological disturbance secondary to the nephrotic state. In this study we therefore compared nephrotic MCNS patients not only to MCNS patients in remission, but also to patients with NS primarily caused by endogenous alterations within the glomerular filter.

Out of a wide range of Type1 and Type2 cytokines, only IL-10 was found to correlate significantly with active MCNS when determined by real-time PCR in PBMC and by ELISA in plasma samples. In accordance with the studies mentioned above, also the expression of IL-13 mRNA in PBMC was increased in nephrotic MCNS patients. However, expression of IL-13 was also upregulated in the NS control group. Therefore, the increased expression of IL-13 in NS may be an epiphenomenon and does not appear to be specific for MCNS.

IL-10 is an 18-kDa non-glycosylated polypeptide secreted by a variety of cells including activated monocytes/macrophages, B cells and T cells, and also epithelial cells such as keratinocytes and bronchial epithelial cells (as reviewed in [26]). IL-10 is member of a family of related cytokines, comprising several cellular homologues including IL-19, IL-20, IL-22, IL-24 and IL-26 and a series of herpes- and poxviral members [27]. It modulates both Th1 and Th2 responses by pleiotropic inhibitory and stimulatory effects on leukocytes. IL-10 suppresses inflammation by reduction of MHC Class II expression and inhibition of production of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1 and IL-12. On the other hand, IL-10 acts as a costimulator of the growth of B-cells, thymocytes and mast cells. In addition, IL-10 counteracts allergic inflammatory reactions by actions on eosinophils and Th2 cells [28]. IL-10 has direct effects also on non-immune cells, such as vascular smooth muscle cells [29], mesangial cells [30] and bronchial epithelial cells [31]. In mice, transgenic overexpression of IL-10 in the lung causes mucus hypersecretion of bronchial epithelial cells [32]. Interestingly, the action of IL-10 in these mice is dependent on the presence of IL-13.

Inflammation is absent in the glomeruli of MCNS patients. Possible effects of IL-10 on the glomerular filtration barrier would therefore consist of direct effects on glomerular cells. The biological effects of cytokines are mediated through cell surface receptors, which transduce the binding of their cognate ligands into cytoplasmic signals that subsequently trigger a cascade of intracellular responses. We earlier showed that podocytes express functional receptors for IL-4 and IL-13 [12]. In the present study, we found that podocytes in vivo also expressed the heterodimeric receptor for IL-10, consisting of IL-10R1 and IL-10R2, confirming earlier studies on podocytes in vitro [33]. The expression was present both in normal kidneys and in kidney biopsies from nephrotic patients. Incubation with IL-10 enhanced STAT3 and STAT5 signaling in podocytes in vitro, indeed suggesting the presence of a functional IL-10 receptor complex on podocytes.

In conclusion, these studies demonstrate that proteinuria in MCNS is associated with an elevated expression of IL-10 by PBMC, which may cause signaling in podocytes. Our study does not allow identifying the trigger of IL-10 overexpression in MCNS patients. Together with the studies mentioned above, it might again reflect an alteration in the immune balance that occurs during relapse. It is unclear whether in those patients showing overexpression, IL-10 is responsible or required for
proteinuria. Pharmacokinetical and pharmacodynamical studies on the administration of human recombinant IL-10 to healthy volunteers have not reported on the development of NS in these individuals [34]. While IL-10 itself may not be sufficient to induce disease in healthy volunteers, other factors in addition to IL-10 in patients may be responsible for the development of NS. A multifactorial etiology with involvement of multiple cytokines and chemokines has been established for Th2-associated diseases such as asthma and atopic dermatitis; yet therapeutic strategies aimed at one of the cytokines involved have shown promising results [35, 36]. Large prospective multicenter studies are warranted to unravel the factors involved in the pathogenesis of MCNS.

Acknowledgments

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References