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# IL-8 production by peripheral blood mononuclear cells in nephrotic patients

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**IL-8 production by peripheral blood mononuclear cells in nephrotic patients.** We studied the interleukin 8 (IL-8) gene expression by peripheral blood mononuclear cells (PBMC) and the IL-8 serum concentration in patients with idiopathic minimal lesion nephrotic syndrome (IMLNS) and other glomerulopathies. PBMC from eight of the nine (IMLNS) patients in relapse demonstrated the presence of IL-8 mRNA. All three IMLNS patients in remission ($P = 0.0026$ when compared to patients in relapse) and the two patients with nephrotic syndrome with other glomerulopathies failed to elicit an IL-8 mRNA response. Eleven of the 12 IMLNS patients in relapse showed IL-8 serum concentration above the level of detection. Only one of the seven patients in remission had detectable serum levels of IL-8 ($P = 0.0033$ when compared to levels from IMLNS patients in relapse). IL-8 serum levels were not detectable in three patients with nephrotic syndrome and other glomerulopathies. Supernatants of PBMC cultures from IMLNS patients in relapse increased the $^{35}$sulfate uptake by rat GBM. This effect was abolished by the addition of anti-IL-8 neutralizing antibody to the culture media and reproduced by the addition to the media of IL-8 in concentrations found in the serum of IMLNS patients in relapse. Finally, the effect of IL-8 on the $^{35}$sulfate turnover of the glomerular basement membrane (GBM) sulfated compounds was evaluated *in vitro*. A significant decrease in the percentage of residual $^{35}$sulfate incorporated in the GBM (41 ± 5, mean ± SEM) was observed in cultures treated with IL-8 as compared to those that were not treated with IL-8 (58 ± 8, $P < 0.01$). Because IL-8 affects the metabolism of GBM compounds that may play a role in glomerular permeability, this lymphokine may have a potential pathogenic role in the proteinuria of IMLNS.

Cytokines are hormone-like proteins produced by lymphocytes and monocytes that regulate the function of the immune system. Cytokines are thought to play a role in the pathogenesis of the idiopathic minimal lesion nephrotic syndrome (IMLNS) [1]. Soluble products of peripheral blood mononuclear cells (PBMC) may mediate the increased glomerular permeability to plasma proteins. Circulating factors and supernatants from IMLNS PBMC cultures when injected into the renal artery have been shown to increase glomerular permeability to albumin [2] and to decrease the cationic stains of the capillary wall [3].

Interleukin 8 (IL-8) is a cytokine originally purified from the supernatant of human monocytes stimulated with lipopolysaccharide [4] but also known to be secreted by lymphocytes, endothelial, and tubular cells. IL-8 was primarily thought to be a neutrophil chemotactic factor. Subsequently, it has been shown to activate neutrophil function and may serve as a secondary mediator of inflammation. Recently, from a variety of recombinant human cytokines tested, we and others have found that IL-8 could alter the metabolism of the rat glomerular basement membrane (GBM) sulfated compounds (unpublished data) [5]. Because this *in vitro* finding may have pathogenic significance in patients with IMLNS, we measured the plasma concentration of IL-8 and assessed the capability of IL-8 production by PBMC of IMLNS patients at different stages of development of the nephrotic syndrome.

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## Methods

### *Patients*

Twenty-four patients with IMLNS defined by the International Study of Kidney Disease in Children [6] and four patients with nephrotic syndrome and other glomerulopathies were included in the study. Fifteen were males and thirteen were females. Their ages ranged from three to 20 years with a median age of 10 years.

Patients with ILMNS were studied in remission and in relapse. Remission was defined as serum albumin within normal limits and normal urinary protein excretion (trace or negative by Albustix® or <5 mg/m$^2$/hour). Patients were considered in relapse if massive proteinuria (3+ by Albustix or >50 mg/kg/day) and a low serum albumin level (< 25 g/liter) were present.

### *Detection of circulating interleukin 8*

The presence of IL-8 in serum samples of patients with IMLNS and other glomerulopathies was measured by radioimmunoassay using a commercially available kit (R and D Systems Inc., Minneapolis, Minnesota, USA). All samples were studied in duplicate.

### *Northern blot analysis*

To detect the presence of mRNA for IL-8, Northern blot analysis was performed on PBMC collected by Ficoll Hypaque density gradient centrifugation of heparinized whole blood [7] from nine patients with IMLNS in relapse, three patients in remission, and two patients with other glomerulopathies (focal segmental glomerulosclerosis and membranous nephropathy) immediately after PBMC cells were collected. In four of the

same patients the mRNA expression for IL-8 was also studied after 20 hours of incubation in RPMI 1640 medium.

Total cellular RNA was isolated according to the method of Chomczynski and Sacchi [8]. Twenty microgram samples of RNA were fractionated on a 0.8% agarose gel, transferred to Nytran filter paper and then stained with methylene blue-acetate to determine presence and integrity of transferred RNA. Hybridization was performed using a random-primed IL-8 cDNA probe labeled with dCTP-$^{32}$P [9].

### *Effect of anti-IL-8 antibody on PBMC mediated increase in $^{35}$sulfate uptake by rat glomerular basement membrane (GBM)*

Glomeruli from Sprague-Dawley rats (100 to 350 g) (Charles River, Wilmington, Massachusetts, USA) were isolated by the method described by Fong and Drummond [10]. At the end of the procedure, a drop of the preparation on a glass slide was examined for purity by counting and expressing as a percentage of the total number of glomeruli and nonglomerular fragments. Preparations that were more than 5% contaminated with tubular fragments were discarded.

Glomeruli obtained from each rat were suspended in 6 ml of Basal Medium Eagle (BME, without sulfate, Catalog no: 424–1300, Grand Island Biological Company, New York, New York, USA) and divided into three aliquots. One was cultured with 3 ml of supernatants from PBMC cultures from IMLNS patients in relapse, another was cultured with 3 ml of same supernatant but with the addition of 40 μg/ml of anti-human IL-8 neutralizing IgG antibody (R and D Systems Inc.), and 3 ml of BME was added to the third aliquot. This sample served as the control.

The culture medium was supplemented with 200 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% decomplemented fetal calf serum, and 80 μCi of $^{35}$sulfate (carrier free; New England Nuclear, Boston, Massachusetts, USA). Glomeruli were incubated at 35°C in Petri dishes (50 × 50 mm) in a humidified atmosphere of 5% $CO_2$. Twenty-four hours later, samples were centrifuged, supernatant discarded and pellet lyophilized. The pellet was kept at −70°C until GBM was isolated.

### *Isolation of the GBM*

GBM was prepared from lyophilized, isolated glomeruli by the method of Meezan et al [11] with minor modifications. The washed precipitate was dried and resuspended in Atomlight (New England Nuclear); radioactivity was counted for one minute in a Beckman liquid scintillation counter. Results were expressed in counts per minutes (cpm) per milligram of dry glomerular weight.

### *Effect of recombinant human monocyte derived IL-8 on $^{35}$sulfate uptake by rat GBM*

Glomeruli obtained from each rat were divided into two aliquots. IL-8 was added to one and the other was cultured alone. Two concentrations (200 and 10.000 pg/ml) were used. Samples, after the addition of 80 μCi $^{35}$sulfate, were incubated for 24 hours as previously described. Glomeruli were lyophilized, GBM isolated, and radioactivity counted.

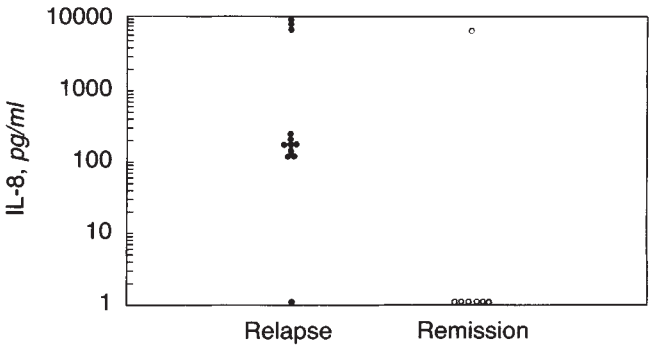


**Fig. 1.** *Serum IL-8 (pg/ml) in IMLNS patients in relapse and in remission.*

### *Effect of IL-8 on the $^{35}$sulfate turnover of GBM sulfated compounds*

Glomeruli obtained from each rat were divided into four aliquots. Glomeruli were incubated in 5 ml of BME supplemented with 80 μCi of $^{35}$sulfate. Recombinant human IL-8 (200 ng/ml) was added to two of the four aliquots.

After 24 hours of incubation, samples were centrifuged, supernatants discarded and pellet washed twice and resuspended in the same culture medium but without the radioactive sulfate. Once again IL-8 (200 ng/ml) was added to the same previous cultures that received IL-8, but 1 mM of $MgSO_4$ was added to one of the cultures supplemented with IL-8 and to one of the cultures which did not have IL-8. All samples were incubated for 12 more hours.

At the end of incubation the samples were centrifuged, the supernatants were collected, and the pellet was lyophilized and kept at −35°C until the GBM was isolated. Radioactivity was counted on isolated GBM. Results were expressed in cpm per milligram of dry glomerular weight.

### *Statistics*

Statistical analysis was performed using Fisher exact test, Wilcoxon matched-pairs signed-rank test, Friedman analysis of variance for ranks with the family-wise error maintained at 0.05 with the use of Holm's stepdown multiple comparison procedure, and 2 × 2 repeated measures analysis of variance.

## Results

Figure 1 depicts the IL-8 serum concentration in IMLNS patients in relapse and in remission. Eleven of the 12 patients whose IL-8 serum concentration was measured during relapse showed values above the level of detection. Only one of the seven patients studied during remission had a detectable IL-8 serum concentration ($P < 0.0033$ as compared to relapse patients). This patient went into relapse one week later. All others remained in remission for at least three months after IL-8 was measured. IL-8 serum levels were not detectable in three patients with nephrotic syndrome associated with other glomerulopathies (data not shown).

The expression of the IL-8 gene by PBMC was studied in nine IMLNS patients during relapse, three IMLNS patients during remission, and two patients with other glomerulopathies (focal glomerulosclerosis and membranous nephropathy; Fig. 2A). Data were collected from two sets of experiments. Figure 2B

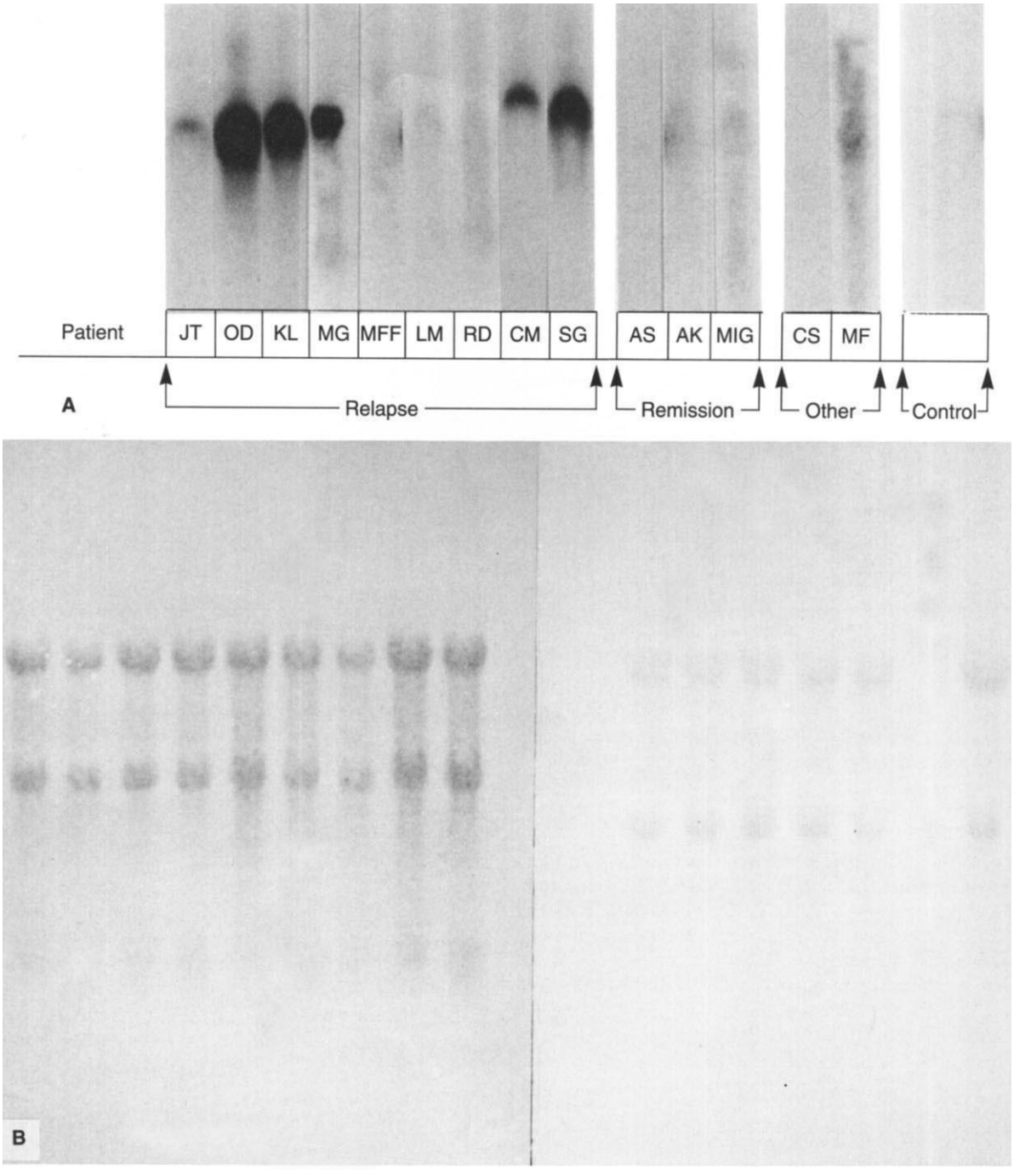


**Fig. 2A.** *Spontaneous IL-8 mRNA induction by lymphocytes from IMLNS patients in relapse and in remission.* At 0 hour IL-8 specific mRNA was detected by Northern blotting (see text). IMLNS patients are represented in relapse and in remission. Other depicts patients with glomerulopathies other than IMLNS. Control shows results of lymphocytes from a healthy adult. **B.** Methylene blue-acetate stained filter of 20 $\mu$g of total RNA extracted from patients' PBMC and a normal control. The 28s and 18s ribosomal bands are shown.

shows the methylene blue-acetate stained Nytran filters after loading each lane with 20 $\mu$g of total RNA extracted from patient's PBMC. The 28s and 18s ribosomal bands from all the lanes verified that comparable amounts of total RNA were loaded for each lane. PBMC from eight of the nine patients in relapse demonstrated the IL-8 mRNA. The only (MFF) patient who had a weak reaction at 0 hour showed a strong mRNA expression after 24 hours of incubation. All three patients in remission failed to elicit an IL-8 mRNA response at 0 hour ($P$ = 0.0026 as compared to IL-8 mRNA response of IMLNS patients in relapse). In the two patients with nephrotic syndrome associated with other glomerulopathies (CS and MF) no IL-8 and mRNA response was detected.

The spontaneous IL-8 mRNA induction at 0 and 20 hours by

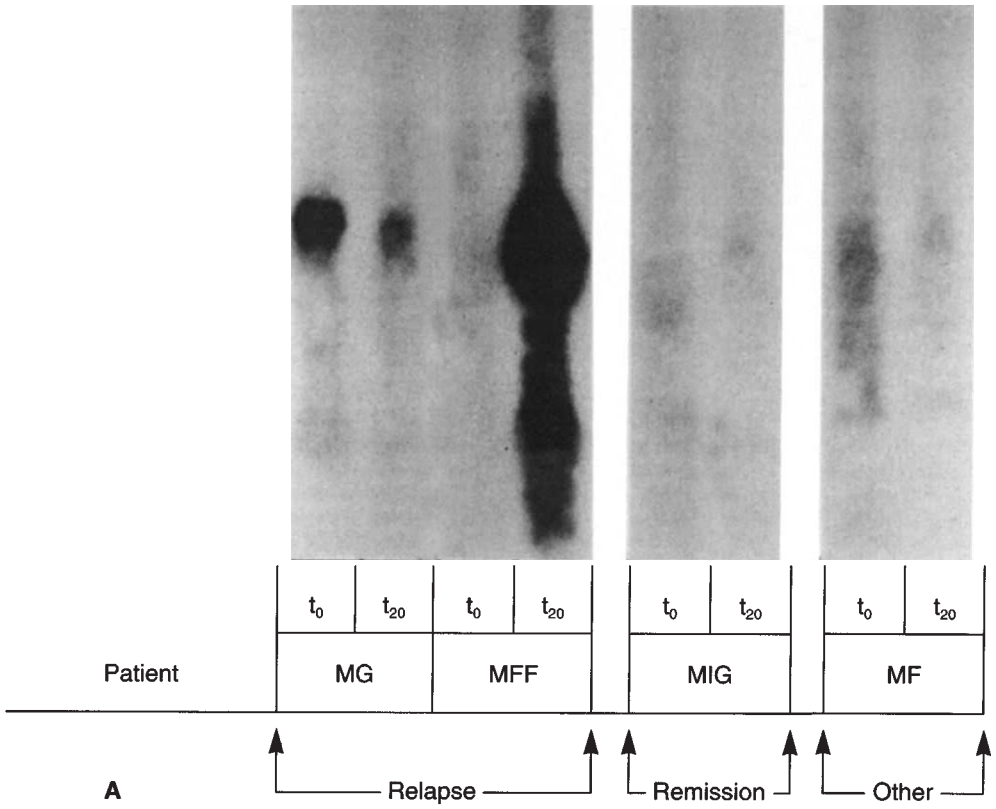


**Fig. 3A.** *Spontaneous IL-8 mRNA inductions at 0 and 20 hours by lymphocytes from IMLNS patients in remission (MIG), in relapse (MG, MFF) and other glomerulopathy (MF).* **B.** Methylene blue-acetate stained filter of 20 μg of total RNA extracted from patients' PBMC. The 28s and 18s ribosomal bands are shown.

PBMC from IMLNS patients in remission (MIG), in relapse (MG and MFF), and another glomerulopathy (MF) is shown in Figure 3A. Figure 3B shows the methylene blue-acetate stained filter of 20 μg of total RNA extracted from PBMC of same patients. Figure 3 demonstrates that comparable amounts of total RNA were loaded in each lane. Only the two IMLNS patients in relapse demonstrated the presence of IL-8 mRNA after 20 hours of incubation.

The effect of two different concentrations of human monocyte derived IL-8 in $^{35}$sulfate incorporation by rat GBM is depicted in Table 1. At both concentrations, the $^{35}$sulfate uptake in rat GBM was significantly higher when glomeruli were cultured with IL-8 than when glomeruli were cultured alone.

The effect of anti-IL-8 neutralizing antibody on supernatant PBMC cultures mediated increase $^{35}$sulfate uptake in rat GBM is shown in Table 2. The $^{35}$sulfate incorporation in rat GBM was significantly higher when glomeruli were cultured with supernatant from nine IMLNS patients in relapse (800 ± 229 cpm/mg dry glomerular weight; mean ± SEM) than when glomeruli were cultured with supernatants from the same patients and the addition of anti-IL-8 antibody (400 ± 91 cpm/mg; mean ± SEM; $P = 0.009$) or glomeruli were cultured alone (442 ± 94 cpm/mg; mean ± SEM; $P = 0.009$). No significant differences in $^{35}$sulfate uptake were observed when glomeruli alone cultures were compared to glomeruli incubated with supernatant from IMLNS patients and the addition of anti-IL-8 antibody ($P = 0.48$).

The effect of IL-8 on the $^{35}$sulfate turnover of GBM sulfated compounds is shown in Table 3. $^{35}$Sulfate incorporation was significantly higher when glomeruli were cultured with the addition of IL-8 (413 ± 43 cpm/mg dry glomerular weight; mean ± SEM) than it was when glomeruli were cultured in medium alone (314 ± 39 cpm/mg mean ± SEM; $P < 0.05$). Furthermore, the addition of 1 mM of $MgSO_4$ to the culture media was associated with a significant decrease in $^{35}$sulfate uptake in GBM of both IL-8 treated and medium alone cultures as compared to the uptake observed in cultures without $MgSO_4$ ($P < 0.0005$ and $<0.001$, respectively), confirming the effect of the chase (nontagged) sulfate molecule in the reincorporation of the radiolabeled sulfate into the GBM sulfated compound. Finally, the percentage of residual $^{35}$sulfate incorporated in the GBM after 12 hours of incubation with $MgSO_4$ were 41 ± 5 (mean ± SEM) for cultures stimulated with IL-8 and 58 ± 8 for glomeruli cultured in the absence of IL-8 ($P < 0.01$), suggesting a higher $^{35}$sulfate turnover in the IL-8 treated cultures. Moreover, when the same data were analyzed using F test for interaction in a 2 × 2 repeated measures analysis of variance, once again there was a significant difference between the changes observed after Mg was added to the culture media in both experimental conditions ($F = 9.35$; $P = 0.012$).

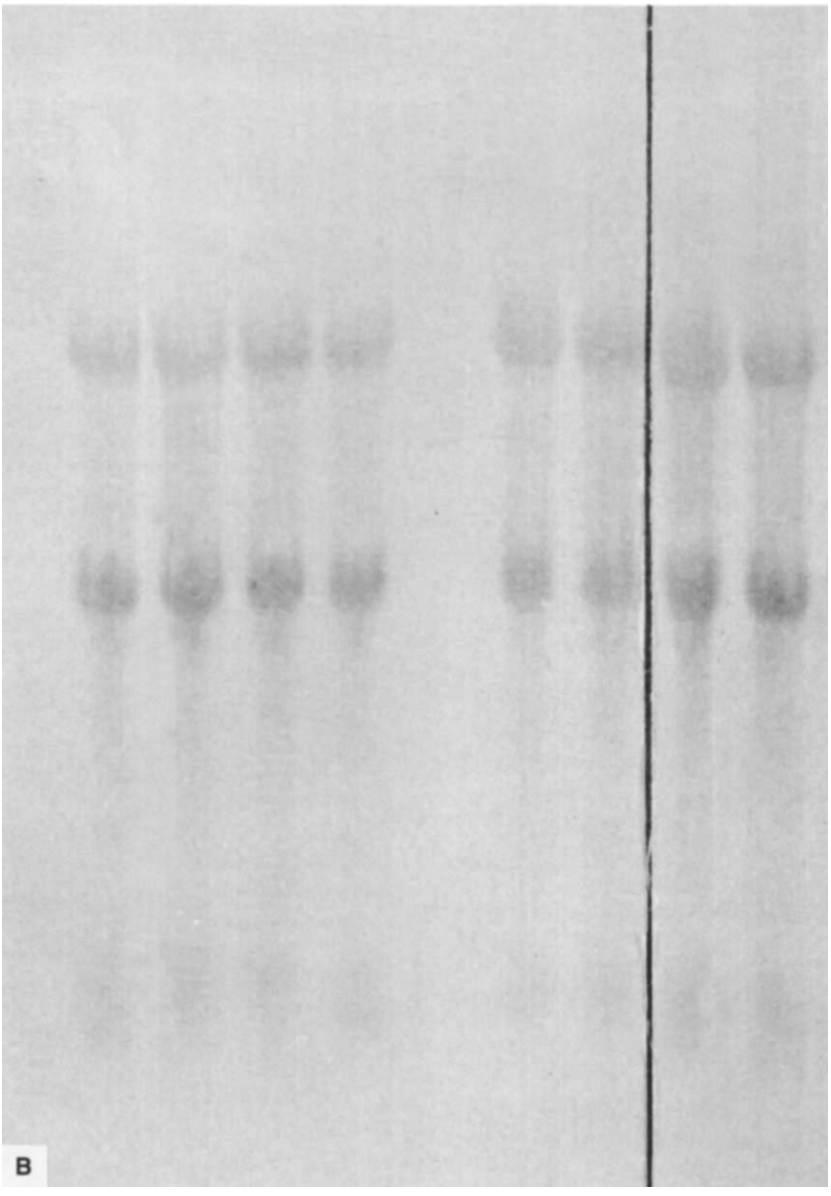


**Fig. 3.** *Continued*

## Discussion

Our data show that IL-8 serum concentrations are increased in IMLNS patients in relapse. All but one of the patients in remission had levels not detectable by the radioimmunoassay. However, one week later this patient was found to have mild edema, proteinuria, and low serum albumin, indicating that the increased IL-8 serum concentration had preceded the development of proteinuria. Furthermore, the elevated serum levels seem to be restricted to IMLNS patients since the three patients with proteinuria associated with other glomerulopathies had undetectable serum levels of IL-8.

IL-8 is secreted by monocytes or lymphocytes [12] and other nonimmune types of human cells. These include proximal and distal tubular [13] and endothelial cells [14]. In this study, we present evidence that PBMC from IMLNS patients in relapse can express and secrete IL-8. This increase release of IL-8 by PBMC could cause, at least in part, the elevated IL-8 serum levels observed in these patients.

The reason for the spontaneous increase in IL-8 production by PBMC in these patients is not known. It does not seem to be secondary to the nephrotic state, since patients with other types of nephrotic syndrome did not have detectable IL-8 in their serum. Most nephrotic patients' relapses are preceded by a respiratory tract infection [15]. This perhaps triggers the gene expression for IL-8. However, this hypothesis cannot be confirmed because no data are available on the effect of viral infections on PBMC IL-8 production.

An unexpected finding in this study was the presence of mRNA for IL-8 after PBMC from IMLNS patients in relapse were incubated for 20 hours. In the original studies on IL-8

**Table 1.** $^{35}$Sulfate uptake by GBM after incubation of glomeruli with two concentrations of human, monocyte-derived IL-8

| | (A) Glomeruli + IL-8 | (B) Glomeruli alone |
|---|---|---|
| (a) IL-8 *200 pg/ml*[a] | 617 | 379 |
| | 1117 | 1095 |
| | 1242 | 698 |
| | 1962 | 1707 |
| | 1870 | 1552 |
| | 2035 | 1435 |
| | 1216 | 732 |
| | 833 | 504 |
| | 854 | 446 |
| $\bar{X}$ ± SEM | 1305 ± 176 | 948 ± 170 |
| | (A′) Glomeruli + IL-8 | (B′) Glomeruli alone |
| (b) IL-8 *10,000 pg/ml*[b] | 2077 | 789 |
| | 490 | 224 |
| | 919 | 466 |
| | 923 | 836 |
| | 331 | 357 |
| | 978 | 784 |
| | 3072 | 1265 |
| | 1500 | 1051 |
| | 838 | 698 |
| $\bar{X}$ ± SEM | 1236 ± 287 | 719 ± 110 |

$^{35}$Sulfate uptake by rat GBM (cpm/mg dry glomerular weight)
[a] A vs. B, $P = 0.009$
[b] A′ vs. B′, $P = 0.034$

**Table 2.** Effect of anti-IL-8 neutralizing antibody on PBMC mediated increased $^{35}$sulfate uptake by rat GBM

| | (A) Glomeruli and PBMC | (B) Glomeruli and PBMC and anti-IL-8 | (C) Glomeruli alone |
|---|---|---|---|
| | 806 | 708 | 532 |
| | 985 | 874 | 909 |
| | 1446 | 342 | 702 |
| | 2253 | 398 | 496 |
| | 650 | 554 | 630 |
| | 211 | 50 | 130 |
| | 142 | 115 | 124 |
| | 305 | 202 | 179 |
| | 406 | 367 | 272 |
| $\bar{X}$ ± SEM | 800 ± 229 | 400 ± 91 | 442 ± 94 |

$^{35}$Sulfate uptake by rat GBM (cpm/mg dry glomerular weight).
$X_v^2 = 13.8$; $P < 0.01$.
A vs. B, $P = 0.009$; A vs. C, $P = 0.009$; B vs. C, $P = 0.48$.

secretion, when normal monocytes were stimulated with LPS, the mRNA IL-8 production was shut down after a few hours despite persistence of the stimulus in the culture media [4]. In our patients, the gene associated with the increased production of mRNA for IL-8 did not down-regulate after 20 hours in culture, despite the absence of a known stimulus for IL-8 secretion of IL-8 and its increased serum concentration.

There are scarce data on serum concentration of lymphokines in patients with nephrotic syndrome. In a group of patients with IMLNS, membranoproliferative glomerulopathy and diabetic nephropathy, Suranyi et al found that only tumor necrosis factor (TNF) plasma concentrations were elevated with respect to normal controls. Plasma levels of IL-1, IL-2 and interferons (IFN A and B) were not different than those observed in normal

**Table 3.** $^{35}$Sulfate GBM uptake of glomeruli after cold chase

| A Glomeruli + IL-8 | B Glomeruli + IL-8 + $MgSO_4$ | C Glomeruli alone | D Glomeruli alone + $MgSO_4$ |
|---|---|---|---|
| 602 | 288 | 302 | 100 |
| 331 | 117 | 282 | 141 |
| 174 | 129 | 141 | 120 |
| 523 | 195 | 219 | 69 |
| 324 | 89 | 447 | 170 |
| 562 | 120 | 427 | 141 |
| 417 | 224 | 363 | 437 |
| 447 | 269 | 214 | 182 |
| 427 | 204 | 316 | 219 |
| 532 | 97 | 569 | 192 |
| 207 | 65 | 176 | 110 |
| Mean ± SEM | | | |
| 413 ± 43 | 163 ± 23 | 314 ± 39 | 171 ± 30 |

Glomeruli cultured *in vitro* in the presence (A) or absence (C) of IL-8 received a 12 hour pulse of $^{35}$sulfate, as indicated in **Methods**, washed with incubation media and incubated 12 hours in medium containing unlabeled sulfate (1 mM $MgSO_4$) without isotope (B and D). $^{35}$Sulfate GBM uptake (cpm/mg dry glomerular wt).
A vs. B, $P < 0.0005$; A vs. C, $P = 0.023$; C vs. D, $P = 0.001$.

controls [16]. Moreover, renal tubular cells (one of the cells known to produce IL-8) have been shown to express IL-8 mRNA and secrete antigenic IL-8 after stimulation with TNF [13]. Perhaps TNF can also stimulate the production of IL-8 by nephrotic PBMC.

Schnaper has determined that urine and serum from certain nephrotic patients contain a suppressor lymphokine (soluble immune response suppressor, SIRS) that inhibits antibody production and delayed type hypersensitivity response *in vivo* [17]. SIRS is produced by lymphocytes from patients with IMLNS and other glomerulopathies, and also by patients with other diseases who are not nephrotic. Furthermore, there is no evidence to support a role for SIRS in mediating proteinuria in nephrotic syndrome [17].

IL-2, IL-4 and SIRS are lymphokines found in the supernatant of PBMC cultures from patients with IMLNS and other glomerulopathies [18–20]. Their role in the development of proteinuria in IMLNS is unknown. Because all these lymphokines have been detected in patients with nephrotic syndrome other than IMLNS, it is currently thought that they play a secondary role, if any, in the increased glomerular permeability to plasma proteins. Moreover, the significance of these *in vitro* findings is questionable because, as in the case of IL-2, PBMC from nephrotic patients have been shown to release IL-2 in the culture media [20] but no increase in serum IL-8 concentration has been reported [16].

Previous studies have hypothesized a role for lymphokines in the proteinuria of IMLNS. In this report, we have shown that PBMC from IMLNS patients in relapse express IL-8 mRNA and that only IMLNS patients in relapse had an increased IL-8 serum concentration.

Furthermore, it seems that the previously described [21] *in vitro* effect of the PBMC supernatants from IMLNS patients in relapse is mediated, at least in part, by IL-8 because: (a) IL-8 was detected in the supernatants of the PBMC cultures from these patients (data not shown); (b) this effect was abolished by the addition of anti-IL-8 neutralizing antibody to the culture

media; and (c) the addition of IL-8 to the culture media in concentrations found in the serum of IMLNS patient in relapse also increased the $^{35}$sulfate uptake by the GBM.

IL-8 found in the serum of the IMLNS patients in relapse may play a role in the metabolism of glomerular sulfated compounds since some IL-8 concentrations observed in the serum of these patients were found *in vitro* to increase the $^{35}$sulfate uptake by rat GBM.

Finally, our *in vitro* experiments demonstrate that the augmented GBM $^{35}$sulfate incorporation associated with IL-8 or the supernatants from IMLNS PBMC cultures [22] is secondary to an increased $^{35}$sulfate turnover of the GBM sulfated compounds.

Polyanionic molecules in the GBM have been demonstrated to restrict the passage of anionic serum proteins to the urinary space by creating a charge selective barrier. This barrier seems to play a major role in the glomerular permeability to serum albumin in IMLNS. The nature of this electrostatic screen has not been defined. Sulfate from heparan sulfate proteoglycan and carboxylic groups from other molecules in the GBM might be the key components. Although our results do not prove IL-8 to be the pathogenetic lymphokine in IMLNS, these studies demonstrating its presence in the serum of patients during relapse and its effect on sulfate turnover in the glomerular basement membrane, albeit indirect, suggest a possible relationship between IL-8 and increased glomerular permeability to proteins. Further studies including the chronic infusion of IL-8 in the renal artery of experimental animals are required to evaluate the potential of IL-8 as a proteinuria inducing lymphokine in IMLNS.

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