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Interleukin 6 (IL-6) in serum and urine of renal transplant recipients

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SUMMARY

Hybridoma growth factor (HGF) is a 20–25 kD protein, supporting the growth of hybridoma cells *in vitro* and capable of replacing feeder cells. It was shown to be produced by human monocytes and a number of cultured cell lines. Recently, HGF was found to be identical to interferon-$\beta 2$ or 26 kD protein and BSF-2, and was renamed interleukin 6 (IL-6). Using a sensitive bio-assay we were able to measure IL-6 activity in the serum and urine of healthy volunteers and renal transplant recipients. Low levels of IL-6 were present in the serum but not in the urine of healthy individuals. In contrast, both serum and urine of renal transplant recipients contained high levels of IL-6 directly after transplantation and during acute rejection episodes. On the basis of kinetic studies of the IL-6 response, it is concluded that serial measurement of IL-6, especially in urine, may be of value in monitoring renal transplant recipients. Moreover, the sensitivity of the bioassay will allow for detailed studies as to the biological significance of IL-6 in health and disease.

Keywords IL-6 body fluids renal transplant recipients

INTRODUCTION

In 1980, Astaldi *et al.* described the presence in supernatants from cultured human endothelial cells of a factor supporting the growth of hybridoma cells *in vitro* and capable of replacing feeder cells (Astaldi *et al.*, 1980). A sensitive assay for this growth factor was developed by Lansdorp *et al.* (1986) using a murine antibody-producing B cell hybridoma that for its growth is absolutely dependent on the presence of what was then called the hybridoma growth factor (HGF). It was found that human monocytes are by far the most potent producers of HGF. Much weaker sources of HGF are cultured fibroblasts, smooth muscle cells and endothelial cells (Lansdorp *et al.*, 1986). Further studies revealed that HGF is a protein with a molecular weight of 21–25 kD on SDS polyacrylamide gel electrophoresis; chromatofocussing yielded two peaks of activity at pI 4·9 and 5·1, respectively. Hybridoma growth factor was found to be clearly dissimilar from interleukin 2 and 3 and interferon-α and γ (Aarden, Lansdorp & De Groot, 1985).

Because preliminary experiments suggested that HGF might be related to interleukin 1 (IL-1), we investigated whether HGF could be detected in serum or urine of healthy human volunteers and renal transplant recipients. This was done not only to gain more insight into the possible biological significance of HGF *in vivo*, but also to see whether HGF measurements could be useful for monitoring renal transplant recipients.

Recently, Aarden *et al.* have succeeded in cloning the gene for HGF and expressing it in *Escherichia coli* (1987). The sequence of HGF proved to be identical to that very recently described for interferon-$\beta 2$ or 26 kD protein (Haegeman *et al.*, 1986; Zilberstein *et al.*, 1986), as well as to the sequence of BSF-2, a B cell differentiation factor, reported by Hirano *et al.* (1986). For this molecule, the name interleukin 6 (IL-6) has been proposed (Poupart *et al.*, 1987).

MATERIALS AND METHODS

Patients

Renal transplant recipients were all treated with cyclosporin A (CyA) as the sole immunosuppressive drug. CyA was started directly pre-operatively, 12 mg/kg/day; this dose was decreased stepwise according to the CyA whole-blood levels. These were aimed at 400–800 ng/ml in the early post-transplant period and at 150–400 ng/ml later on. Acute rejection episodes, defined both clinically and by fine needle aspiration cytology and/or histology, were treated by bolus injections of 500 mg of methylprednisolone i.v. for 6 days.

Healthy volunteers were recruited from the medical staff as well as from blood-bank donors.

Serum

Serum was separated from freshly drawn blood, heat-treated at 56°C for 30 min and stored at −20°C. In initial experiments, it

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was ascertained that the heating procedure, necessary to remove nonspecific toxicity from the serum, did not influence HGF activity. Sera were tested within 2 months of collection.

Urine

Urine was collected as fresh 0900 h voids. After centrifugation at 1000 ***g*** for 10 min, nonspecific low molecular-weight inhibitors of the HGF assay were removed by filtration, using a YM-10 filter (cut-off point 10,000 mol.wt; Amicon, Lexington, MA). After filtration, the samples were reconstituted to the original volume with phosphate-buffered saline. In all filtration procedures, a standard IL-6 preparation, purified as described (Aarden *et al.*, 1985), was run in parallel as a control. In these controls, IL-6 activity after filtration was $95 \pm 4\%$ ($n = 40$) of the activity before filtration. Filtered samples were stored at $-20°C$ and tested within 2 months of collection.

IL-6 assay

To increase the sensitivity of the assay, a variant of the original IL-6-dependent cell line (B 13·29) was isolated using subcloning in decreasing amounts of IL-6. This variant subclone 9·9 enables the measurement of IL-6 produced by a single human monocyte in 100 μl of medium (Aarden *et al.*, 1987). The 9·9 hybridoma cells, maintained in culture as described by Lansdorp *et al.* (1986), were harvested and washed twice in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal calf serum (FCS). Cells (5000) in 100 μl of IMDM-5% FCS were cultured together with a titration of the material to be assayed in a final volume of 200 μl in flat-bottom microtitre plates (Costar, cat. no. 3596). After 44 h of culture, 0·2 μCi ^{3}H-thymidine (Radiochemical Centre, Amersham, UK, spec. act. 2 Ci/mmol) was added. Four hours later, the cells were harvested and ^{3}H-thymidine incorporation into DNA was measured as described by Du Bois *et al.* (1974).

In all assays, a titration curve of a standard IL-6 preparation (Aarden *et al.*, 1985) was included. Interleukin 6 activity resulting in half the maximal ^{3}H-thymidine incorporation of the standard preparation was arbitrarily defined as one unit. All determinations were performed in triplicate. Under the conditions described above, the assay was not influenced by the CyA and methylprednisolone concentrations occurring *in vivo*.

RESULTS

IL-6 in serum and urine of healthy volunteers

As can be seen from Fig. 1 in all healthy individuals tested, low levels of IL-6 activity could be detected in the serum (mean $7·7 \pm 0·6$ U/ml; $n = 48$). This was in contrast to data obtained with urine, where IL-6 activity exceeded the detection threshold of the assay (1 U/ml) in only three out of 27 cases tested.

In mixing experiments using either standard IL-6 preparations (Aarden *et al.*, 1985) or IL-6-containing urine samples, it could be shown that this lack of IL-6 activity in the urine was not due to the presence of inhibitors (data not shown).

IL-6 in serum and urine of renal transplant recipients

Although in most of the 32 patients tested, the IL-6 level in serum collected immediately before transplantation was within the range of the healthy controls, in about 20% of the patients no IL-6 activity could be detected at all (Fig. 2a). Here too, this was not due to inhibitory substances. This group of patients did

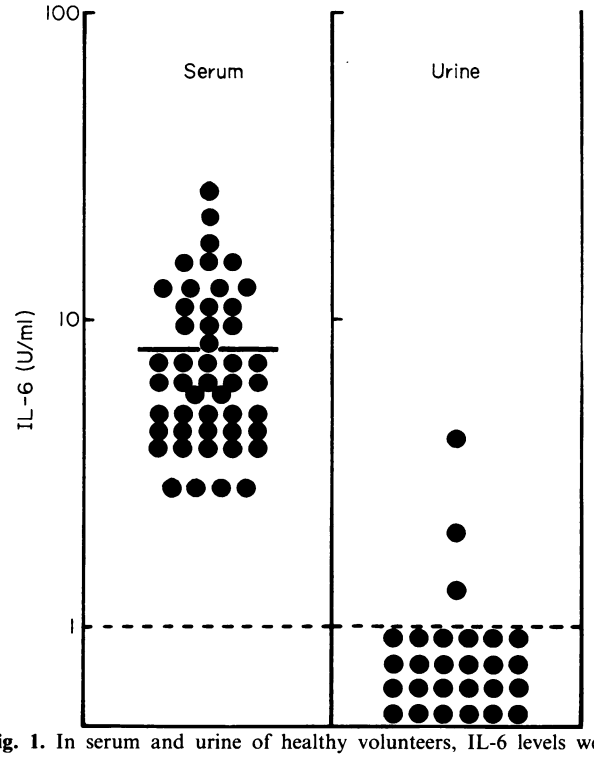

Fig. 1. In serum and urine of healthy volunteers, IL-6 levels were determined as described under Materials and Methods. The horizontal bar indicates the mean value (for serum 7·7 U/ml; s.e.m. 0·6; $n = 48$). The horizontal dotted line indicates the detection threshold of the assay (1 U/ml).

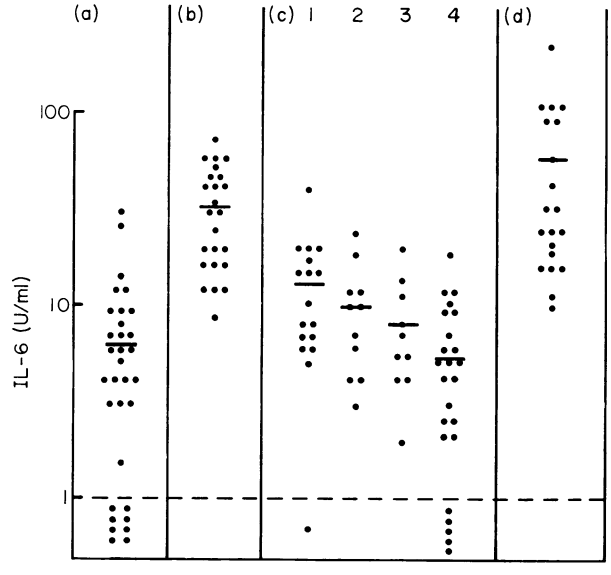

Fig. 2. IL-6 activity in serum of 32 renal transplant recipients in relation to the clinical situation. Mean values (indicated by the horizontal bars) $\pm$ s.e.m. were as follows: (a) pretransplantation: $6·3 \pm 1·2$ U/ml ($n = 32$); (b) 2 days after transplantation: $31·8 \pm 3·6$ U/ml ($n = 24$); (c) stable graft function for: C1, 1–2 weeks after transplantation or rejection: $12·5 \pm 2·1$ U/ml ($n = 17$); C2, 2–3 weeks: $10·0 \pm 1·8$ U/ml ($n = 11$); C3, 3–4 weeks: $8·0 \pm 1·5$ U/ml ($n = 10$); C4, stable for more than 4 weeks: $5·2 \pm 0·9$ U/ml ($n = 24$); (d) 0–3 days before clinical acute rejection: $54·7 \pm 12·6$ U/ml ($n = 20$).

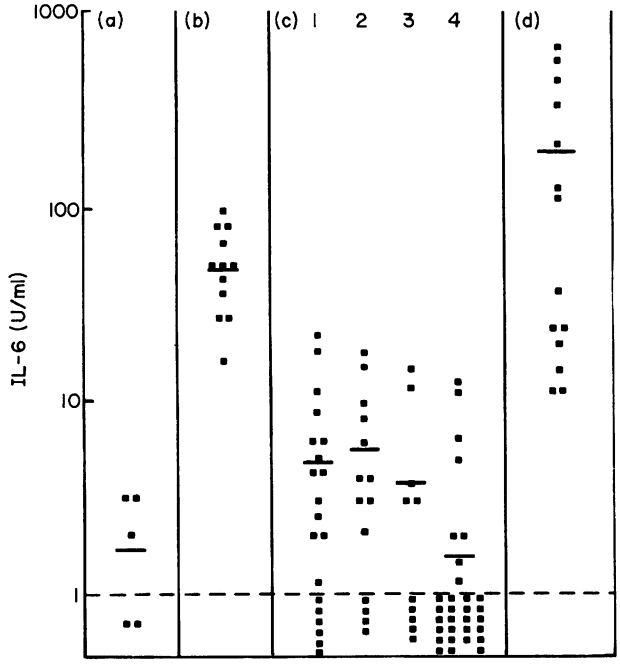

Fig. 3. IL-6 activity in urine of 32 renal transplant recipients in relation to the clinical situation. Mean values (indicated by the horizontal bars) ± s.e.m. were as follows: (a) pretransplantation: 1·7 ± 0·6 U/ml (n = 5); (b) 2 days after transplantation: 49·5 ± 6·6 U/ml (n = 12); (c) stable graft function for: C1, 1–2 weeks after transplantation or rejection: 4·9 ± 1·1 U/ml (n = 20); C2, 2–3 weeks: 5·3 ± 1·5 U/ml (n = 14); C3, 3–4 weeks: 3·7 ± 1·6 U/ml (n = 10); C4, > 4 weeks: 1·5 ± 0·5 U/ml (n = 31); (d) 0–3 days before clinical acute rejection: 182·0 ± 60·0 U/ml (n = 14).

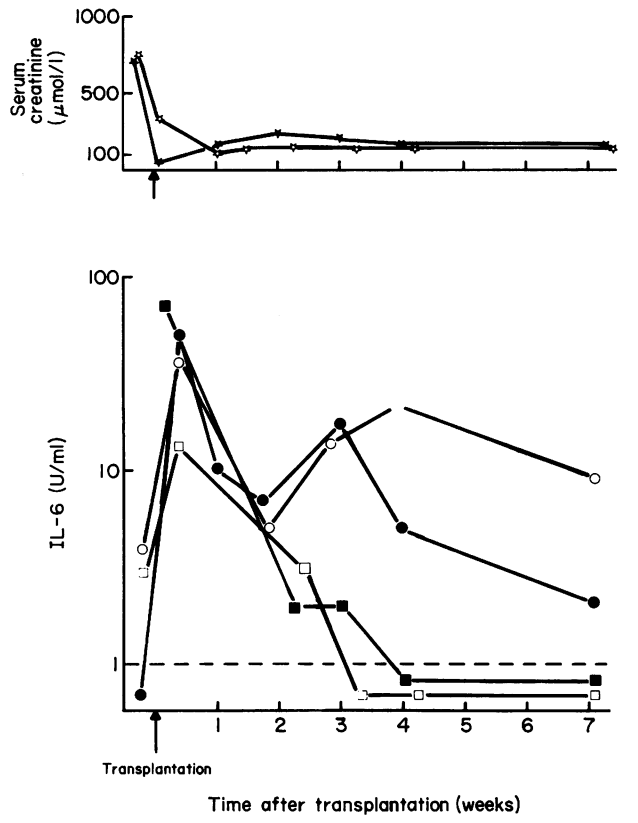

Fig. 4. IL-6 levels in serum and urine from two patients with uncomplicated renal transplantation. Patient A: serum (○), urine (□), patient B: serum (●), urine (■). In the upper part of the figure, the corresponding plasma creatinine levels (in μmol/l) are shown. Patient A (☆); patient B, (★).

not differ from the others with respect to clinical course or outcome (data not shown).

After transplantation, the IL-6 serum levels rose rapidly in all patients, reaching peak levels of up to 100 U/ml on day 2 after transplantation (mean increase 79·6% ± 14·8 (s.e.m.), n = 21) (Fig. 2b). In patients with stable, good functioning grafts, IL-6 levels were within the normal range again (Fig. 2c), with the exception of a small group without detectable IL-6 activity in the serum. This group proved to consist of the individuals also lacking detectable IL-6 activity pretransplantation.

Interestingly, in patients suffering from acute rejection, sera drawn up to 2 days before the clinical diagnosis was made, contained significantly increased IL-6 activity (54·7 ± 12·6 U/ml; n = 20) (Fig. 2d).

As to IL-6 in urine samples of the same group of patients, a similar pattern was found though the differences in relation to the clinical situation were more pronounced: very high levels of IL-6 (up to 1000 U/ml) were present on day 2 after transplantation and during rejection (Fig. 3b and d). The overlap with values obtained during stable, good graft function is minimal. Not surprisingly, data on pretransplant IL-6 activity in urine are limited in number, because most patients were anuric before transplantation. However, high levels (> 10 U/ml) were not observed. To evaluate the prognostic significance in terms of rejection, we studied IL-6 levels in a longitudinal series of serum and urine samples. In uncomplicated transplantations, after the early peak, IL-6 levels declined and returned towards normal within 2 to 3 weeks (Fig. 4). In Fig. 5, IL-6 levels in serum and urine from a patient with 2 early rejections are shown. Here, in the first week after the transplantation, the IL-6 activity remained high, especially in the urine. During successful antirejection therapy with methylprednisolone, there was a rapid fall towards low levels. However, in the weeks following this episode, there was a slow but steady increase of IL-6 activity in the urine. In this period, there were neither clinical nor biochemical signs of rejection. This occurred only at week 5, where another successful antirejection therapy was accompanied by a rapid normalization of IL-6 levels, both in serum and urine.

The observation that IL-6 levels increase both directly after transplantation and during rejection suggested that IL-6 might be an early and nonspecific marker of the inflammatory response, possibly related to the acute phase reaction. Therefore, we studied IL-6 in serum and urine of six gynaecological patients undergoing abdominal surgery for non-malignant diseases. The results, shown in Fig. 6, were similar to those obtained in renal transplant recipients, and confirm the nonspe-

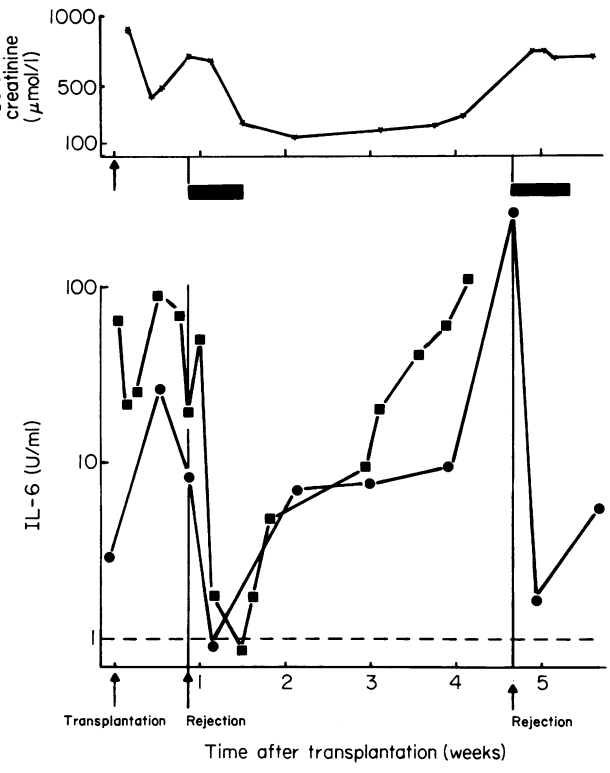

Fig. 5. IL-6 levels in serum (●) and urine (■) of a patient with two early acute rejection episodes. In the upper part of the figure, the corresponding plasma creatinine levels (in μmol/l) are shown. (■) antirejection treatment with MPNS 500 mg i.v. for 6 days.

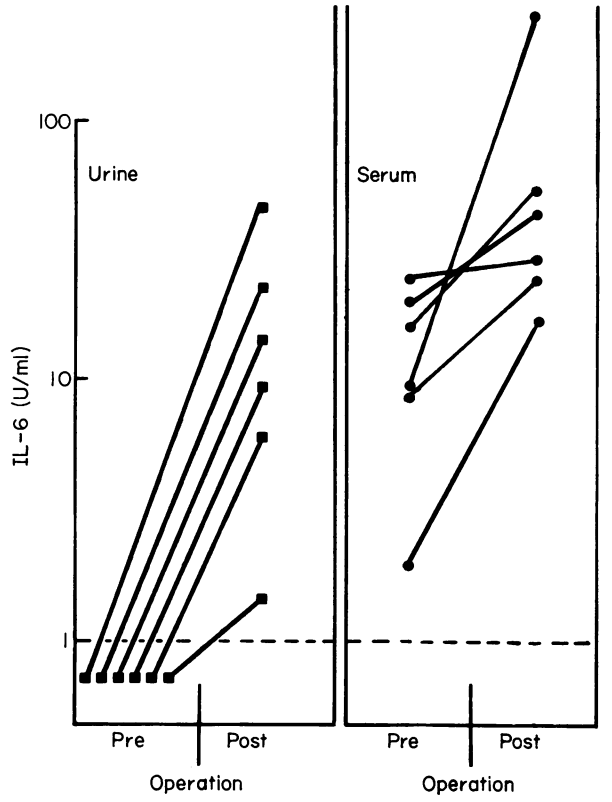

Fig. 6. Serum and urine IL-6 levels in six women directly before and 2 days after abdominal surgery for non-malignant disease.

cific character of the IL-6 response. However, the kinetics studied in one such patient, indicated a more rapid return towards normal IL-6 levels (7 days; data not shown).

Molecular analysis of IL-6 activity

Because IL-6 is measured as sample-induced proliferation of a IL-6-dependent cell line, the data shown above only indicate the presence of IL-6 *activity* in serum and urine. To investigate whether we were measuring native IL-6 (mol.wt 21–25 kD) or either split products retaining biological activity or aggregates, a IL-6-containing urine sample was concentrated and applied to an AcA-54 column as described (Aarden *et al.*, 1985); an IL-6 standard preparation was run in parallel. Fractions were tested in a 1:2 dilution. The results in Fig. 7 clearly show only one peak of IL-6 activity, coinciding with native IL-6. In normal serum, a second peak of activity was noted; the molecular weight, not yet fully characterized, was around 5–7 kD (data not shown). The specificity of the IL-6 measurements was demonstrated by experiments in which both in serum and urine IL-6 activity could be blocked completely by polyclonal rabbit anti-IL-6 antibodies (see Table 1).

DISCUSSION

HGF, a protein with a molecular weight of 21–25 kD and a pI of 4·9 to 5·1 (Aarden *et al.*, 1985) was originally defined according to its capacity to support the growth of hybridoma cells *in vitro* (Astaldi *et al.*, 1980). Very recently, the gene for HGF was cloned and sequenced by Aarden *et al.* (1987). HGF proved to be identical to interferon-β2 (Zilberstein *et al.*, 1986) or the 26 kD molecule (Haegeman *et al.*, 1986) and the B cell differentiation factor BSF-2 (Hirano *et al.*, 1986), so the name interleukin 6 was proposed (Poupart *et al.*, 1987). The sensitivity of the IL-6 bioassay, described by Lansdorp *et al.* (1986), was increased so that the IL-6 production of single cells could be measured. In limiting dilution experiments, it could be demonstrated that within the human mononuclear cell population, only monocytes are capable of IL-6 production (Aarden *et al.*, 1987). Notably normal T cells do not produce detectable amounts of IL-6, not even after stimulation with a variety of stimuli such as solid-phase anti-CD3, PHA, PMA and recombinant IL-1α and β (Helle & Aarden, unpublished). Because BSF-2 was derived from an HTLV-1-infected T cell line (Hirano *et al.*, 1986), it would be very interesting to investigate whether it is possible to induce IL-6 production in normal (non-IL-6-producing) T cells by infecting them with HTLV-1.

In the present study, we have shown that using this sensitive bioassay, it is possible to demonstrate low levels of IL-6 in the serum, but in general not in the urine of healthy humans. In contrast, in renal transplant patients, high levels of IL-6 activity can be detected in serum as well as in urine. Peak values are reached 2 to 3 days after transplantation. In uncomplicated cases, these values return to normal within 3 weeks. In patients experiencing rejection episodes, IL-6 activity increases significantly in the days preceding the clinical manifestations. The increases are most pronounced in urine samples.

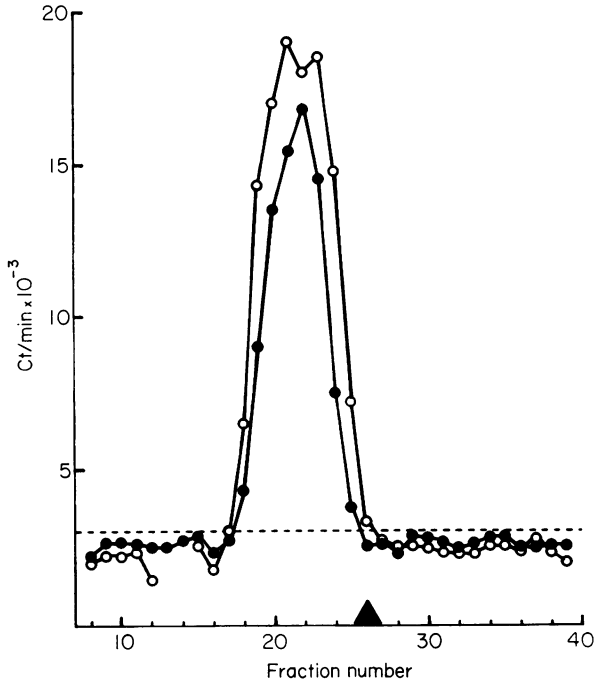

Fig. 7. Molecular analysis of IL-6 activity in urine. IL-6-containing urine (20 U/ml) was concentrated six times by filtration on YM 10. Ten millilitres were applied to an AcA-54 column (100 × 2·5 cm). Fractions of 2 ml were collected and assayed for IL-6 activity at a 1:2 dilution (○). As a control, a similar procedure was applied to a standard IL-6 preparation (●). The dotted line indicates the medium control. (▲) marker molecule (phenol red).

Table 1. Inhibition of IL-6 activity by polyclonal rabbit anti-IL-6 antibodies

Addition		Proliferation of IL-6-dependent cell line (ct/min)	
		−	+anti-IL-6 antiserum (1:100)
Serum	(1:10)	41,300	3800
Urine 1	(1:10)	44,000	1600
2	(1:10)	40,600	2500
3	(1:10)	29,480	2000
Purified IL-6	(1:100)	37,300	3300
Medium		750	850

Serum and urine from three different renal transplant recipients, as well as purified IL-6 (prepared as described under Materials and Methods) were tested for IL-6 activity both in the absence as well as in the presence of polyclonal rabbit anti-IL-6 antibodies. The dilutions of the material tested are given in parentheses.

It is very likely that the increases in urinary IL-6 in renal transplant patients directly after operation and during acute rejection are due to increased production of the IL-6 in the body (as also suggested by parallel increases in serum levels) and do not merely reflect renal dysfunction in the freshly transplanted kidney (e.g. diminished tubular reabsorption or increased glomerular permeability).

This is based on the following arguments: (a) a similar IL-6 response can be observed in surgical patients having a normal renal function; (b) in 18 patients with renal insufficiency either on the basis of interstitial diseases or glomerulopathies (with proteinuria up to 20 g/24 h), no increased urinary IL-6 levels were found; (c) there was no correlation between renal function as measured by the serum creatinine level and IL-6 excretion in the urine (data not shown).

Our study does not allow conclusions as to whether IL-6 is produced locally in the kidney (e.g. by inflammatory monocytes or mesangial cells) or elsewhere in the body.

In mixing experiments, it was repeatedly shown that the dramatic decrease in serum and urine IL-6 levels during methylprednisolone treatment was not due to inhibitory effects of methylprednisolone or its metabolites on the IL-6 assay. Therefore, the decrease probably reflects diminished IL-6 production.

The observation that similar fluctuations in IL-6 levels occur in gynaecology patients undergoing abdominal surgery indicates that the increases in IL-6 activity are not specific for renal transplantation and might be related to the acute phase reaction.

The mol.wt of IL-6 excludes the possibility that IL-6 itself is a known acute phase protein. However, as the main source of IL-6 is the monocyte, and monocytes are known to be involved in the induction of the acute phase response via the production of interleukin 1 (Dinarello, 1984), there might be a functional relationship between IL-1 and IL-6, although both interleukins are clearly dissimilar at the molecular level.

At present, two separate molecules with IL-1 activity have been cloned and produced via recombinant technology, i.e. IL-1α and IL-1β, differing in pI (5·0 and 7·0, respectively) and showing only 26% homology in their amino-acid sequences (March *et al.*, 1985). Probably both molecules are produced as 30 kD precursor proteins; the 17 kD carboxyterminal split products were shown to retain IL-1 activity (March *et al.*, 1985). Both these recombinant products were tested in our HGF assay and shown to be negative. However, purified HGF was active in a number of IL-1 assays, including the thymocyte assay, and assays measuring the induction of the acute phase protein α_2-macrofetoprotein, both *in vivo* when HGF was injected i.v. into rats as well as *in vitro* in rat hepatocyte cultures (to be published). Therefore, it seems possible that at least some of the many biological effects of IL-1 are mediated by IL-6. This is in agreement with the observation by Content *et al.* (1985) and Zilberstein *et al.* (1986) that in fibroblasts mRNA for IL-6 is induced by IL-1. To clarify the exact functional relationship between IL-1 and IL-6, experiments are now in progress aimed at the production of monoclonal anti-IL-6 antibodies.

Although we have presented data indicating that IL-6 measurements might be useful in monitoring renal transplant patients, studies of IL-6 activity in various diseases (such as infections, haematological malignancies and autoimmune disorders) may increase our understanding of the biological

significance of IL-6 *in vivo*, not only as to its role in the acute phase reaction but also in (abnormalities in) the regulation of B-cell differentiation.

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