In a phase 1 study of recombinant interleukin-6 (rIL-6) in patients with advanced solid tumors (n = 15), we discovered that the endogenous IL-6 levels, in pretreatment plasma or serum samples, were distributed into two groups. One set of patients (designated "type 1"; n = 9) was characterized by low plasma IL-6 levels (48 to 1,700 pg/mL) as measured using an enzyme-linked immunosorbent assay (ELISA) for IL-6. In the second set of patients (designated "type 2"; n = 6), IL-6 ELISAs showed high levels of plasma IL-6 (50 to 600 ng/mL). Neither group had detectable B9 hybridoma cell growth factor activity associated with the IL-6 in their pretreatment plasma or serum. Plasma C-reactive protein (CRP) levels were markedly elevated in type II patients suggesting that the circulating IL-6 was biologically active in vivo. In both groups of patients there was a small but significant increase in B9 activity in the plasma within three hours after rIL-6 administration (n = 5). Gel filtration profiles showed that circulating IL-6 in type 1 patients, 15 to 120 minutes after rIL-6 administration was of approximate mass 20 to 40 kD, whereas in type 2 patients, the IL-6 before and after exogenous rIL-6 administration was indistinguishable and was of an approximate mass of 200 kD. IL-6 immunoaffinity purification of the 200 kD complexes showed these to contain multiple isoforms of IL-6 (14 to 31 kD) and the soluble IL-6 receptor (sIL-6R; 50 to 55 kD). A distinguishing clinical history was that all of the type 2 patients had been actively immunized with an anti-idiotypic monoclonal antibody (MoAb) (M1K2-23) 3 to 12 months before initiation of this study for advanced melanoma. An analysis of the plasma IL-6 content in other melanoma patients (n = 16) during anti-idiotypic MoAb immunization indicated that marked up to 600 ng/mL and sustained (several months) elevations of circulating "chaperoned" IL-6 were induced by active immunization regimens.

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a murine anti-idiotypic MoAb (MK2-23), which bears the internal image of a human high-molecular-weight melanoma-associated antigen (HMW-MAA). Biochemically, this IL-6 was in 200-kD complexes that included sIL-6R. Longitudinal studies of plasma IL-6 levels in such patients confirmed that the dramatic elevations of circulating chaperoned IL-6 were sustained for long periods (over several months) particularly in malignant melanoma patients treated with immunotherapy. These data identify what may well be a broad class of patients in whom high-molecular-mass complexes consisting of “chaperoned” IL-6 routinely exist in the peripheral circulation at high concentrations.

**MATERIALS AND METHODS**

**Plasma and serum samples.** Blood samples were derived from 15 patients with advanced solid tumors enrolled in a phase I study of SDZ ILS 969 (recombinant human IL-6 from E. coli) with ICE chemotherapy performed in the Division of Oncology, Department of Medicine, New York Medical College (Valhalla, NY). The protocol used was approved by the Institutional Human Subjects Review Board. rIL-6 (SDZ ILS 969) was provided by the Cytokine Development Unit, SANDOZ PHARMACEUTICAL CORPORATION (East Hanover, NJ). Detailed descriptions of the study protocol, the clinical parameters studied and the clinical outcome are described elsewhere (Mittelman et al., in preparation). Briefly, patients were enrolled in an open-label study consisting of three 28-day cycles of chemotherapy. In cycle 1, they received ICE chemotherapy intravenously on days 1 through 3, followed by a rest day (day 4), then 14 days of SDZ ILS 969 subcutaneously once a day (days 5 to 18) followed by a 10-day washout period. Cycle 2 consisted of ICE alone, and cycle 3 again consisted of ICE plus SDZ ILS 969. Mesna was administered at 1,600 mg/m²/d intravenously in four equally divided doses on cycle days 1 to 3 starting immediately before ifosfamide. Ifosfamide 2,000 mg/m²/d was administered by a 1 to 2 hour intravenous infusion on days 1 to 3, carboplatin 450 mg/m² on day 1, and etoposide 200 mg/m²/d on days 1 to 3. Starting on cycle day 5, patients received rIL-6 (SDZ ILS 969) subcutaneously at a dose of 2.5 to 25 μg/kg body weight for 14 days. The IL-6 dose was escalated in different patients in the range 2.5 to 25 μg/kg body weight depending upon the enrollment sequence of the patient and the patient’s tolerance.

Blood samples for preparation of heparinized plasma and for serum samples were derived from four groups of four patients each (total of 16) who had received the anti-idiotypic MoAb MK2-23, which bears the internal image of HMW-MAA: MoAb MK2-23 (group A), MoAb MK2-23 cross-linked with keyhole limpet hemocyanin (KLH) (group B), MoAb MK2-23 together with Bacillus Calmette Guerin (BCG) (group C), or MoAb MK2-23 cross-linked to KLH together with BCG (group D). In addition sera collected over a 2-year period before enrollment of patient JS in the ICE plus IL-6 phase 1 study were evaluated.

**B9 growth factor assay for IL-6.** The IL-6 bioactivity of heat-inactivated (56°C for 30 minutes) plasma or serum samples was assayed by monitoring their ability to induce proliferation of tumorigenic B9 hybridoma cells using standardized procedures described earlier. The WHO interim reference standard for IL-6 88/514 was included in every assay.

**ELISAs for serum and plasma samples.** ELISAs for IL-6 in serum and plasma samples were performed by the procedure of Kenney et al. as applied to human IL-6. In the two assays, the anti-IL-6 MoAbs (4IL6-H11 or 7IL6-H12) were used as the capture antibodies. In both assays, the reporter biotinylated MoAb 5IL6-H17 was used for detection. The characteristics of these MoAbs and their use and limitations in evaluating different preparations of natural human IL-6 as well as rIL-6 preparations have been described elsewhere. Human E.coli-derived rIL-6 interim reference preparation 88/514 and the World Health Organization reference standard for IL-6 (89/548) were used for calibration of the assays.

**Sephadex G-200 gel filtration chromatography.** Plasma samples (0.4 to 0.8 mL) were fractionated through a Sephadex G-200 column (2.5 × 60 cm; (V,)= 55 mL; excluded volume (V,)= 164 mL; included volume (V)= V, + V, as described earlier. Eluate fractions of 2.5 mL were collected after the first V, and the elution continued for an additional two V, (≥ 50 fractions). All eluted fractions were calibrated for molecular size by the use of the following marker proteins: ribonuclease-14 kD, chymotrypsinogen A-26 kD, ovalbumin-46 kD, aldolase-158 kD, and ferritin-440 kD. The Sephadex G-200 and the calibrating proteins were purchased from Pharmacia LKB (Piscataway, NJ).

**Preparation of recombinant human sIL-6 receptor (sIL-6R) and rabbit polyclonal antibody to sIL-6R.** A soluble form of the IL-6R (p80) was engineered for expression in Spodoptera frugiperda (Sf21 insect cells) by placing a truncated cDNA corresponding to the human IL-6R coding region, but lacking the transmembrane sequence, downstream of the wild-type polyhedron promoter in plasmid pVL1393. In this vector, the first polyhedron ATG is mutated to allow the use of the inserted gene’s starting ATG, while maintaining the use of the polyhedron polyadenylation signal downstream of the inserted IL-6R cDNA sequence. For this construction, plasmid p236, containing the entire coding region of the human IL-6R was digested with Sph I and Ssp I to generate a 1.1-kb fragment that contained the coding sequence for 342 amino acid residues starting from the first ATG through Asn-342 of the IL-6R. Asn-342 is sixteen amino acids N-terminal to the beginning of the transmembrane region of the IL-6R. This DNA fragment was then ligated into the multiple cloning site of pVL1393 that had been previously digested with Sma I and Bgl II. Transfection of this engineered plasmid into Sf21 insect cells, produced high-titer virus stocks and production of rIL-6R was performed as described in the Baculovirus Expression System Bulletin. High levels of baculovirus vector expression leading to secretion of recombinant human sIL-6R into the culture medium was obtained in Sf21 cells that had been adapted to serum-free conditions. Recombinant sIL-6R was purified by passage of the culture medium through an immunoaffinity column containing immobilized anti-IL-6R MoAb MT-18. Methods for preparation of the immunoaffinity column and its use in protein purification have been previously described. Purity and concentration of recombinant sIL-6R preparations were established by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using commercial protein standards (Pharmacia, Piscataway, NJ).

Anti-sIL-6R serum was obtained from New Zealand white rabbits immunized with four biweekly subcutaneous injections of purified rIL-6R (20 μg per injection) with Freund’s adjuvant. PAGE and Western blot analyses. IL-6 and sIL-6R present in plasma preparations were analyzed by immunoaffinity or gel filtration chromatography followed by SDS-PAGE and immunoblotting. Aliquots of IL-6 and sIL-6R preparations and various test samples were suspended in Laemmli buffer, electrophoresed through an SDS-polyacrylamide gel (15% w/vol) under reducing conditions.
and transferred to polyvinylidene difluoride (PVDF) membranes (Schleicher and Schuell, Keene, NH) according to the method of Towbin et al. An immunoperoxidase procedure (Vectastain, Elite kit, Vector Laboratories Inc, Burlingame, CA) was used to detect both IL-6 and sIL-6R using a 1:500 dilution of rabbit antihuman IL-6 or sIL-6R antiserum, respectively.

**Measurements of serum C-reactive protein.** Serum CRP levels were assayed using the method of rocket immunoelectrophoresis.

**Statistical analyses.** All statistical analyses were performed using TRUE EPISSTAT (Epistat Services, Richardson, Texas) software.

**RESULTS**

In studies of the biochemistry of IL-6 in human serum, a technical concern has been the possibility that the observations may be the result of proteolysis or other consequences of clotting and clot retraction. Therefore, both serum and plasma samples were prepared from blood derived from patients enrolled in the phase 1 rIL-6 study. Most of the IL-6 data from the phase 1 study reported in this article were derived from plasma samples. Parallel analyses of serum samples taken at the same time as the plasma samples provided results largely similar to those obtained using plasma. For simplicity of presentation only data obtained using plasma are described.

**Plasma levels of IL-6 after the administration of rIL-6.**

Levels of plasma IL-6 after rIL-6 injection were measured using three assays: (1) 4IL6/5IL6 ELISA, (2) 7IL6/5IL6 ELISA, and (3) B9 cell growth factor activity. Two sandwich ELISAs represent distinct ELISAs that have been found to distinguish between different antigenic forms of IL-6. The 7IL6/5IL6 ELISA is highly sensitive to rIL-6 (sensitivity 1 to 10 pg/mL) and was expected to detect any exogenously derived rIL-6 present in serum or plasma samples. In contrast, the 4IL6/5IL6 ELISA is relatively insensitive toward rIL-6 (sensitivity 100 to 200 pg/mL), but preferentially recognizes native circulating high-molecular-mass IL-6.

The B9 hybridoma growth factor assay is very widely used as a bioassay to determine levels of IL-6 present in human plasma and serum. Figure 1 illustrates the results obtained when these three assays were used to measure IL-6 levels in plasma obtained from patients with solid tumors before administration of rIL-6. The results show that the three assays measured different levels of IL-6 in plasma. In addition, Fig 1 illustrates an unexpected observation. Plasma IL-6 levels measured using either ELISA distribute themselves into two discrete sets: type 1 (solid symbols) corresponding to \( \geq 10^3 \) pg/mL of IL-6 as measured in either ELISA, and type 2 (open symbols) corresponding to \( \geq 10^5 \) pg/mL of IL-6 in the 4IL6/5IL6 ELISA or \( \geq 10^4 \) pg/mL of IL-6 in the 7IL6/5IL6 ELISA. The disparity between type 1 and 2 is only partially indicated because half the plasma samples from type 1 patients fell below detection limits of each IL-6 ELISA and have been arbitrarily placed at those limits. Despite the presence of very high IL-6 antigen levels, only two plasma samples had barely detectable levels of B9 cell growth factor activity. All of the patients in the type 2 group had a prior history of active specific immunotherapy for melanoma (see below).

Changes in circulating IL-6 levels following subcutaneous administration of rIL-6 were investigated. Figure 2A and B show plasma IL-6 concentrations in patients at the indicated times after the administration of rIL-6. For the sake of clarity, the data have been separated into two panels that represent plasma IL-6 concentrations as measured in the B9 bioassay and in the 7IL6/5IL6 ELISA in patients with high (\( \geq 10^3 \) pg/mL of IL-6; panel A) or low (\( \leq 10^2 \) pg/mL of IL-6; panel B) endogenous IL-6 as shown in Fig 1. In Fig 2, only the 7IL6/5IL6 ELISA data are shown because this assay is routinely at least an order of magnitude more sensitive to rIL-6 than the 4IL6/5IL6 ELISA. In both low (type 1) and high (type 2) patients, IL-6 as measured in the B9 cell growth assay (solid symbols) was near maximal by 30 to 60 minutes after rIL-6 administration (between 10 and 75 U/mL). However, the highest B9 activity levels measured were small. In contrast, the 7IL6/5IL6 ELISA yielded different results in the two kinds of patients. In type 1 patients (low endogenous IL-6), IL-6 ELISA reactivity (solid symbols in Fig 2B) followed a pattern parallel to that of the B9 bioassay results. In type 2 patients (high endogenous IL-6), little or no change was observed in IL-6 ELISA reactivity in plasma after rIL-6 administration (Fig 2A). The appearance of circulating B9
growth factor activity after subcutaneous injection of rIL-6 indicates that at least some of this administered rIL-6 is bioavailable (Fig 2). Circulating B9 activity returns to undetectable levels within 12 to 24 hours after rIL-6 administration (data not shown). The concentration of C-reactive protein (CRP), an acute-phase plasma protein induced by IL-6, was evaluated in several of the type 1 (low) and type 2 (high) plasma samples before rIL-6 administration. Figure 3 shows that the high IL-6 samples had markedly elevated CRP levels, whereas the low IL-6 samples had a lower level of CRP (although still higher than normal values). Although circulating IL-6 in the high patients may be completely devoid of B9 cell growth activity, the data in Fig 3 suggest that it is biologically active in vivo in eliciting an hepatic acute-phase plasma protein response. In low type 1 patients, we have detected substantial increases in CRP levels (to 25 to 30 mg/dL) 24 to 48 hours after subcutaneous injection of rIL-6, indicating that in these patients the liver can be maximally stimulated by the exogenously added rIL-6 (data not shown).

Biochemical characterization of IL-6 in plasma. Figure 4A and B illustrate Sephadex G-200 gel chromatography fractionation of IL-6 plasma samples (1 mL) taken from type 1 or low endogenous IL-6 individuals after rIL-6 administration. As a control, Sephadex G-200 chromatography fractionation of plasma from the same individual before rIL-6 administration showed little detectable 7IL6/5IL6 or 4IL6/5IL6 immunoreactivity in any of the eluted fractions (data not shown). Figure 4 shows that the IL-6 in the plasma of TR and ED eluted off the Sephadex G-200 columns, with a molecular mass of between 10 and 40 kD, consistent with what was expected for monomers or dimers of IL-6. The immunoreactivity was observed only in the 7IL6/5IL6 ELISA (preferential sensitivity to rIL-6) and not the 4IL6/5IL6 ELISA (preferential sensitivity to high-molecular-mass endogenous IL-6). Thus, the data in Fig 4 indicate that the injected rIL-6 remains largely free in the circulation of the low endogenous IL-6 or type 1 patients. In contrast, in a type 2 patient, all of the IL-6 is of high molecular mass (200 kD). Figure 5 illustrates two Sephadex G-200 gel filtration fractionations of plasma (0.5 mL, volume each time) taken from a single individual (JS) before (solid square) and 30 minutes after subcutaneous injection of rIL-6. All of the IL-6 immunoreac-
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Fig 3. CRP levels in the "high" and "low" IL-6 serum samples in Fig 2. CRP concentrations were measured by rocket immunoelectrophoresis and IL-6 by 4IL6/5IL6 ELISA. Solid symbols represent serum samples from patients with no prior history of immunotherapy and open symbols represent patients' serum that have had active cancer immunotherapy. Without making any assumptions about the distribution or relationship about CRP and IL-6 levels this data set achieved a Spearman's correlation coefficient \( r = 0.957 \), Kendall's \( \tau = 0.925 \) and a two-tailed \( P = 0.0000083 \) (using True Epistat statistical software; Epistat Services, Richardson, TX).

Fig 4. Sephadex G-200 column fractionations of plasma from two patients (TR and ED) with "low" endogenous levels of IL-6 at two times after subcutaneous injection of rIL6. Fractionations before injection of rIL-6 are not shown because none of the fractions or starting material contained any detectable (by 7IL6/BIL6 ELISA) IL-6. (A) G-200 fractionation of 0.5 mL samples of plasma from TR taken 15 minutes (58/7IL6) and 60 minutes (62/7IL6) after subcutaneous injection of rIL-6. (B) G-200 fractionation of 0.5 mL samples of plasma from ED taken 60 minutes (106/7IL6) and 120 minutes (108/7IL6) after subcutaneous injection of rIL-6.
Additionally, we have evaluated the plasma and serum sIL-6R levels in all type 1 and 2 patients, using an sIL-6R sandwich ELISA developed in this laboratory, which uses a rabbit polyclonal antibody raised to a baculovirus-derived recombinant human sIL-6R to capture antigen and a biotinylated MoAb to IL-6R as the reporter (unpublished data). The range of sIL-6R levels was between 10 and 30 ng/mL in plasma, with no significant differences among samples from the same patient before and after rIL-6 administration, or among patients subjected to different treatment immunization protocols (data not shown).

Effect of active specific immunotherapy on circulating IL-6 levels. The relationship between active anticancer immunotherapy and circulating IL-6 levels was studied further by retrospective longitudinal monitoring over time of plasma IL-6 levels in patient JS enrolled in the phase 1 rIL-6 study (Fig 7) as well as in an additional group of 16 patients who had been immunized with anti-idiotypic MoAb MK2-23, with or without adjuvants or carriers (Fig 8).

Patient JS, who had metastatic melanoma, had received a combination of the anti-idiotypic MoAb MK2-23 coupled to KLH together with BCG over a 2-year period (Fig 7).25 IL-6 levels were estimated using both the 4IL6/5IL6 and 7IL6/5IL6 ELISAs in plasma samples from JS that had been collected over this 2-year period. The data in Fig 7 show that circulating IL-6 levels in patient JS increased dramatically in response to the immunotherapy and remained at very high levels (up to ≥600 ng/mL) long-term (at least 1 year). It has already been pointed out that plasma CRP levels in patient JS were markedly elevated (≥21 mg/dL) even before the rIL-6 administration, suggesting that the circulating IL-6, which was inactive in the B9 hybridoma growth assay, was bioactive in vivo (Fig 3).

Because JS received a combination MoAb MK2-23 coupled to KLH together with bacillus calmette guerin (BCG), we investigated whether one, two, or all three of these reagents were required for generating persistently elevated levels of IL-6 in blood. Figure 8 follows 7IL6/5IL6 immunoactivity in plasma in four sets of four patients each that had received variations of the same active immunotherapy treatment given to JS. Group A was given only the MoAb MK2-23; group B received MoAb MK2-23 conjugated to KLH; group C was administered MoAb MK2-23 and BCG; and group D received MoAb MK2-23 conjugated to KLH plus BCG.25

It is apparent from Fig 8 that, in general, patients subjected to active specific immunotherapy displayed marked long-term elevations of circulating IL-6 levels. This was observed most consistently in group D, which represents patients receiving the anti-idiotypic MoAb MK2-23 coupled to KLH, with BCG added as adjuvant. Overall, the more aggressive the immunization, the more sustained was the circulating IL-6 response. However, even patients receiving only anti-idiotypic MoAb (see patient A1) showed marked elevations

![Fig 5. Sephadex G-200 column fractionation of plasma IL-6 from a patient (JS) with "high" endogenous levels of IL-6 before (6/7IL6) and 30 minutes after (12/7IL6) subcutaneous injection of rIL-6. A 0.5 mL sample of plasma was fractionated through Sephadex G-200 column and each of the eluted fractions was analyzed for IL-6 content in the 7IL6/5IL6 ELISA.](image1)

![Fig 6. Western blot analyses of 5IL6 MoAb immunoaffinity-purified IL-6 present in the high-molecular-weight fractions (pools of fractions 7 to 16) from the two Sephadex G-200 fractionations (6/7IL6 and 12/7IL6) illustrated in Fig 5. (A) Immunoblot of aliquots of fractions eluted off an anti-IL-6 MoAb immunoaffinity column probed using rabbit anti-rIL-6 antiserum. For comparison, 10 ng of purified human fibroblast IL-6 ("FS-4 IL-6") and of rIL-6 are included. (B) Immunoblot of aliquots of fractions in panel A probed using rabbit anti-rIL-6R antiserum. For comparison, 25 ng of the soluble form of rIL-6R is included.](image2)
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Fig 7. Longitudinal study of IL-6 levels in plasma samples from a patient (JS) during active specific immunotherapy. Upward pointing arrows represent the time of injection (of MoAb MK2-23 + KLH + BCG). Downward pointing arrows represent time of subcutaneous injection of rIL-6 (5 μg/kg of body mass). Measurements of IL-6 by 4IL6/5IL6 ELISA (■) and 7IL6/5IL6 ELISA (△) are illustrated.

Fig 8. Longitudinal study of IL-6 levels in plasma from patients subjected to active specific immunotherapy. (A) 1 through 4, levels of IL-6 in patients before and after injection of MoAb MK2-23. (B) 1 through 4, levels of IL-6 in patients before and after injection of MoAb MK2-23 + KLH. (C) 1 through 4, levels of IL-6 in patients before and after injection of MoAb MK2-23 + BCG. (D) 1 through 4, levels of IL-6 in patients before and after injection of MoAb MK2-23 + KLH + BCG. Upward arrows indicate time of injection. All measurements of IL-6 illustrated were performed using the 7IL6/5IL6 ELISA. Data obtained using the 4IL6/5IL6 ELISA, which were similar, are not shown.

in circulating IL-6 levels with clearly discernable fluctuations down to baseline levels. Twelve plasma samples corresponding to high IL-6 in different patients in Fig 8 were subjected to anti-IL-6 MoAb immunoaffinity column purification and the eluates immunoblotted using rabbit anti-IL-6 or anti-IL-6R antisera. In each case, both IL-6 and sIL-6R were observed in the anti-IL-6 MoAb column-purified eluates (data not shown) indicating that the circulating IL-6 in this selection of samples was in complexes that contained sIL-6R.

DISCUSSION

The salient observation illustrated in this article is the sustained long-term presence of IL-6 in blood at very high concentrations (≥600 ng/mL) in complexes that include sIL-6R in melanoma patients subjected to active specific immunotherapy. The available data raise the possibility that such sustained marked elevations in the levels of circulating "chaperoned" IL-6 is a consequence of active immunization regimens in humans. This unexpected observation complicates the definition of circulating IL-6 levels required to achieve a therapeutic dose of rIL-6. This endogenous plasma IL-6 was (1) immunoreactive in ELISAs and immunoblots, (2) B9 bioassay inactive, (3) in complexes of molecular mass 150 to 200 kD complexes, (4) in association with sIL-6R, and (5) likely to have some biologic activity as CRP levels are concomitantly high. This group of patients with high endogenous plasma IL-6 were melanoma patients who had been previously subjected to active specific immunotherapy that consisted of repeated immunizations with an anti-idio-type MoAb (that mimics the HMW-MAA) cross-linked to a carrier protein KLH and administered with BCG as an
adjuvant.\textsuperscript{17,29} In these type 2 or high endogenous IL-6 patients, the administration of exogenous rIL-6 did not perceptibly alter circulating immunoreactive IL-6 even though levels of B9 bioassay activity did appear in plasma after rIL-6 administration (Fig 2A).

This investigation was initiated with the objective of determining the biochemical state of injected rIL-6 in the peripheral circulation in humans. Previously, we had observed that endogenous IL-6 in humans existed in high-molecular-mass complexes of 150 to 200 and 400 to 500 kD in association with other proteins that included sIL-6R, fragments of CRP, and of complement factors C3 and C4 together with a protein of molecular mass consistent with soluble gp130.\textsuperscript{14} These proteins camouflaged circulating IL-6 such that even very high concentrations of serum IL-6 (1 to 5 pg/mL) registered only picogram/mL levels or not at all in conventional IL-6 ELISAs and in the B9 bioassay.\textsuperscript{14}

In the present study, we observed that rIL-6 injected subcutaneously into patients with low endogenous IL-6 levels appeared rapidly (15 to 120 minutes) in the peripheral circulation in a low molecular mass (10 to 40 kD) form as evaluated by Sephadex G-200 gel filtration analyses (Fig 4). Low levels of circulating B9 assay bioactivity were also observed in rIL-6–injected patients; these were near-maximal by 15 to 60 minutes (Fig 2). We had anticipated that exogenously administered rIL-6 might be distinguished from endogenous circulating IL-6 by SDS-PAGE and immunoblotting because of its increased electrophoretic mobility compared with the posttranslationally modified versions of native human IL-6. Figure 6 shows that endogenous plasma IL-6 included truncated versions of IL-6 of a molecular mass of 15 to 21 kD by SDS-PAGE that were indistinguishable in their electrophoretic mobility from exogenous rIL-6.\textsuperscript{14} Thus, the fate of rIL-6 injected into type 2 patients remains unclear, although we suspect that in these patients all of the rIL-6 is contained in the 150 to 200 kD complexes (Fig 5). Little or no free 10 to 40 kD IL-6 was detected in plasma from type 2 patients (Fig 5) as it was from type 1 patients (Fig 4).

Although inactive in the B9 hybridoma growth bioassay when tested ex vivo, the 200-kD IL-6 complexes retained bioactivity in Hep3B hepatoma cells in culture (data not shown) and in vivo as assayed by the relationship between IL-6 levels and plasma CRP concentrations (Fig 3). Thus, high levels of circulating IL-6 complexes may serve a chaperone function by altering the biologic activity and the pharmacokinetics of IL-6 in vivo. We have previously shown that anti–IL-6 MoAb can serve a chaperone function in vivo in mice and in baboons holding IL-6 in complexes of a mass of 200 kD that can then elicit hepatic effects depending upon the precise molar ratio of IL-6 to chaperone.\textsuperscript{15} In the present studies in humans, we have unequivocally identified sIL-6R as part of the endogenous chaperoned 200 kD complex. It is likely that this complex contains additional proteins such as soluble gp130, fragments of CRP as well as fragments of complement factors C3 and C4. We have recently developed an ELISA to assay IL-6/sIL-6R complexes in plasma/serum using an anti–IL-6R polyclonal antibody as the capture antibody and a biotinylated anti-IL-6 MoAb as the reporter. In preliminary experiments (not shown), this assay has been used to confirm the presence of IL-6/sIL-6R complexes in plasma of patient JS in a manner similar to that illustrated in Figs 5 to 7. Additionally, we have evaluated the sIL-6R levels in plasma and serum independent of IL-6 and found that the levels varied between 10 and 30 ng/mL in all of the patients involved in the current phase 1 study (data not shown). There was no increase or decrease associated with any patient’s condition nor with immunization protocol. Based on these data we infer that in type 2 patients, all of the sIL-6R is associated with IL-6 in a nonstoichiometric fashion with IL-6 levels (>30 picomoles/mL) far exceeding the sIL-6R levels (0.2 to 0.5 picomoles/mL). We interpret this to mean that whereas the IL-6–associated complexes contain sIL-6R, the interaction between IL-6 and other plasma proteins must be essential for complex formation and/or continued circulation of IL-6 in the bloodstream. We are currently evaluating the role of CRP, complement factor fragment C3c, as well as soluble gp130 in the formation of IL-6–associated complexes.

The demonstration that melanoma patients subjected to active specific immunotherapy exhibit high levels of circulating “chaperoned” IL-6 in a sustained long-term fashion (Figs 7 and 8) was unexpected. What is it about aggressive active immunization that leads to this biochemical event? What are the biologic consequences of long-term high-level circulating IL-6? To what extent does this IL-6 contribute to the clinical benefits that might be observed using such immunotherapeutic regimens? To begin to address these questions, we evaluated IL-6 levels in different groups of patients who had been subjected to forms of the same immunotherapy variably augmented with carrier protein KLH and/or BCG as an adjuvant (Fig 8). A simple interpretation of the available data is that the more aggressive the immunization protocol (longer duration or inclusion of carrier or adjuvant like BCG and KLH), the more sustained the elevations in circulating IL-6. The data clearly identify a class of patients in whom very high levels of plasma IL-6 can be expected to occur over long periods of time. These data show that our previous description\textsuperscript{14} of serum IL-6 concentrations in the 1 to 5 μg/mL range in samples from a post-bone marrow transplant patient with intercurrent bacterial infections is a more general phenomenon that can be expected to occur in other defined clinical situations.

Finally, it is now evident from this study as well as from previous data from this laboratory\textsuperscript{14,15} that the many available assays for IL-6 in human plasma/serum do not provide a complete description of circulating IL-6. The very widely used B9 or other hybridoma cell growth assays routinely fail to detect the bulk of circulating human IL-6. Commercial ELISAs for IL-6 are inadequately characterized for their sensitivity to high molecular-mass IL-6 routinely encountered in human plasma/serum. In fact, when cross-compared with Western blottable IL-6 using an antiserum after purification of the plasma/serum IL-6 through anti–IL-6 MoAb immunoaffinity columns, even the two ELISAs for IL-6 used in this study (4IL6/SIL6 and 7IL6/SIL6) underestimate the concentrations of endogenous human IL-6 in unfractionated plasma/serum (Figs 5 and 6). As a practical matter, we routinely use several different assays for IL-6 simultaneously.
until such time that we understand the biochemical of how IL-6 exists in human blood more completely.

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Sustained high levels of circulating chaperoned interleukin-6 after active specific cancer immunotherapy

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