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Characterization of the Heparin-Binding Properties of IL-6[1]

Rosemary S. Mummery and Christopher C. Rider[2]

We establish, using an ELISA approach, that recombinant human and murine IL-6 bind to an immobilized heparin-BSA complex. In the case of human IL-6, this binding is displaceable by soluble heparin, IC_{50} ~2 μg/ml, corresponding to ~200 nM. This binding is specific because chondroitin sulfates B and C fail to compete, whereas chondroitin sulfate A and several heparan sulfates are weak inhibitors. Of a range of chemically modified heparins examined, the strongest competitor was the 2-*O*-desulfated product, but even this showed a considerably reduced IC_{50} (~30 μg/ml). The epitopes of five IL-6-specific mAbs were still accessible in heparin-bound IL-6, and the dimer formed from the association of rIL-6 with its truncated soluble receptor polypeptide, srIL-6α, still bound to heparin. Further analysis showed that heparin competed partially and weakly with the binding of srIL-6 to IL-6; however, it competed strongly for the binding of the rIL-6/srIL-6Rα dimer, to soluble glycoprotein 130. In studies of the proliferation of IL-6-sensitive Ba/F3 cells expressing glycoprotein 130, we were unable to detect any effect of either the removal of cell surface heparan sulfate, or addition of soluble heparin. By contrast, heparin was able to protect IL-6 from digestion by the bacterial endoproteinase Lys-C. Overall, our findings show that IL-6 is a heparin-binding cytokine. This interaction will tend to retain IL-6 close to its sites of secretion in the tissues by binding to heparin-like glycosaminoglycans, thus favoring a paracrine mode of activity. Moreover, this binding may serve to protect the IL-6 from proteolytic degradation. *The Journal of Immunology,* **2000, 165: 5671–5679.**

Human IL-6 (hIL-6)[3] is a soluble cytokine, M_r 26 kDa, with a polypeptide chain length of 183–185 residues (reviewed in Ref. 1). Expression of hIL-6 is induced by a broad range of stimuli in various cell types, including B and T lymphocytes, monocytes, fibroblasts, keratinocytes, bone marrow stromal cells, endothelial cells, and astrocytes. The diverse range of biological activities attributed to hIL-6 includes the proliferation and differentiation of B and T lymphocytes; induction of acute phase protein expression in hepatocytes; proliferation of multilineage hemopoietic stem cells; stimulation of osteoclast production and activity (2); and promotion of neuronal survival, differentiation, and regeneration after injury (3).

Mice homozygous for IL-6 gene deletion show normal lymphoid cell development, but reduced IgG and cytotoxic T cell responses to viral infection. Moreover, acute-phase protein responses to a variety of stimuli are markedly reduced (4). However, such mice also demonstrate an antiinflammatory role for IL-6, because compared with control animals they show larger inflammatory cytokine responses in the airways after exposure to aerosols of endotoxin, and have poorer survival rates after injection of high doses of endotoxin (5). Doubly transgenic mice, overexpressing both hIL-6 and its specific receptor polypeptide, IL-6Rα (CD126), show a gross enlargement of liver and spleen due to extramedullary hemopoiesis, which is not seen in the singly transgenic animals (6). Double transgenics also show accelerated nerve regeneration after axotomy (7). IL-6 expression is elevated in a number of pathological conditions, and indeed its overexpression appears to be a key factor in the maintenance and progression of several chronic diseases. In particular, there is clear evidence for a central role for IL-6 in murine models of rheumatoid arthritis (8, 9), and Castleman's disease, a benign hyperplasia of lymph nodes (10). IL-6 is also strongly implicated in multiple myeloma, not only as an autocrine growth factor for myeloma cells (11–13), but also in the development of the osteolytic lesions associated with this condition (14).

High resolution x-ray (15) and nuclear magnetic resonance studies (16) confirm that hIL-6 is a four α-helical bundle cytokine with two extensive loop regions, one between helices A and B, and the other between C and D. In common with the cytokines cardiotrophin, ciliary neurotrophic factor, leukemia inhibition factor, oncostatin M, and IL-11, IL-6 uses glycoprotein 130 (gp130) as the signal-transducing polypeptide in its high affinity cell surface receptor complex (17). IL-6 initially binds its specific cell surface receptor, IL-6Rα, a polypeptide of M_r 80 kDa, and the resulting heterodimer, then engages gp130 (18). Subsequently, a hexameric complex involving two polypeptides each of IL-6, IL-6Rα, and gp130 is assembled (19), and the dimerization of gp130 within this complex gives rise to signal transduction. In a variation of this receptor-engagement pathway, IL-6Rα also exists as a soluble protein, which functions as a receptor agonist by permitting IL-6 activity in IL-6Rα^-/gp130$^+$ cells (20).

An increasing number of cytokines and ILs are now known to bind to glycosaminoglycans (GAGs) of the heparin and heparan sulfate (HS) family. These are highly acidic, linear polysaccharides that are widely distributed in the extracellular matrix and on cell surfaces (21, 22). This binding is likely to retain cytokines, which are otherwise small, readily diffusible proteins, close to their sites of release in the tissues, thus favoring paracrine rather than endocrine activity. Because many cytokines, including IL-6, are highly pleiotropic, interaction with heparin/HS in the vicinity of their secretion represents a mechanism of regulation, because the cytokine would be mostly available only to neighboring target cells. However, some release of the cytokine into the circulatory

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[3] Abbreviations used in this paper: h, human; FGF, fibroblast growth factor; GAG, glycosaminoglycan; gp, glycoprotein; HS, heparan sulfate; m, murine; s, soluble.

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system could still occur, either because the binding interactions are noncovalent and reversible, or because of the fragmentation of the proteoglycan macromolecule involved by the action of proteases or heparinases. In the latter case, the cytokine could remain bound to GAG-peptide fragments or GAG oligosaccharides.

Beyond such a general role in compartmentalizing cytokines within the tissues, the binding of cytokines to heparin/HS has been shown to have particular roles for individual cytokines. Thus, the binding of fibroblast growth factor-2 (FGF-2) to heparin/HS has been shown to have an essential role in its signaling pathway, because it is a prerequisite for subsequent engagement of the high affinity cell surface FGF-2 receptor (23, 24). In the case of IFN-γ, binding to heparin results in an approximate 100-fold increase in circulatory $t_{1/2}$, as C-terminal proteolysis associated with uptake is blocked (25). These two examples illustrate that the binding of a cytokine to heparin/HS may have major consequences on cytokine activity, and that these may be quite different from cytokine to another.

In this laboratory, we have been investigating the GAG-binding properties of the various ILs to establish the importance of this phenomenon in the regulation of immune responses. For this purpose, we have developed an ELISA approach employing a synthetic heparin-BSA complex as a solid phase. Using this approach, we have characterized the interactions of rIL-2 (26) and rIL-12 (27) with heparin and HS. In the present study, we have employed this ELISA approach to establish that recombinant human and murine IL-6 also bind to heparin and HS. We have characterized this interaction, and examined that effect of the binding of IL-6 to heparin on its interactions with its receptor polypeptides.

Materials and Methods

Reagents and materials

The routinely employed heparin from porcine intestinal mucosal heparin (sodium salt, grade I-A), together with chondroitin sulfates A, B, and C, bovine HS from kidney and intestinal mucosa, and fucoidan were all purchased from Sigma (Poole, Dorset, U.K.). Low and high sulfated porcine HSs HSA and HSE, as described previously (28), were the kind gift of Dr. B. Mulloy (National Institute for Biological Standards and Control, Hertfordshire, U.K.), as were a series of chemically modified bovine lung heparins that we have also fully described elsewhere (29), as series B. rhIL-6 expressed in *Escherichia coli* was routinely purchased from Amersham Pharmacia Biotech (Amersham, Bucks, U.K.), but was also obtained from R&D Systems Europe (Abingdon, Oxon, U.K.). Recombinant murine IL-6 (rmIL-6) and IL-3, also expressed in *E. coli*, were purchased from R&D Systems Europe. Human soluble rIL-6Rα (srIL-6Rα) and human srgp130, both expressed in *Sf* 21 insect cells, together with goat Abs specific for hIL-6, mIL-6, and human srIL-6Rα, were also purchased from the same source. Rabbit anti-goat IgG was obtained from Sigma. The IL-6-specific mAbs 8, 12, 14, 15, and 16 (30) were kindly provided by Dr. Lucien Aarden (CLB, Amsterdam, The Netherlands), and the IL-6R-specific mAb 17.6 (31) was the kind gift of Dr. Daniella Novick (Weitzmann Institute, Rehovot, Israel). Gentamycin, *p*-nitrophenol phosphate substrate tablets, and endoproteinase Lys-C (EC 3.4.21.50) were obtained from Sigma. FCS (European Union-approved grade), RPMI 1640, and L-glutamine solution were obtained from Life Technologies (Paisley, U.K.). Ba/F3-hgp130 cells were kindly supplied by V. Barton and Dr. J. Heath, Department of Biochemistry, University of Birmingham (Birmingham, U.K.).

Heparin-binding ELISA

A covalent heparin-BSA complex was synthesized by coupling heparin chains via their reducing ends to the protein using sodium-cyanoborohydride, as described fully elsewhere (26), except that the reaction mixture contained 34 mg BSA (equivalent to ~0.5 μmol) and 910 mg heparin (equivalent to ~75 μmol). The separation of the resulting complex from unconjugated reactants was achieved by gel filtration on Sepharose 4B (Pharmacia Biotech, St. Albans, Herts, U.K.), also as previously described (26). Mock-conjugated BSA was prepared simultaneously following the same procedures, except that heparin was omitted from the conjugation reaction.

For heparin-binding ELISA (26), wells were coated with 100 μl 50 mM Tris-HCl buffer, pH 7.4, containing 12.7 mM EDTA, and either 5 ng heparin-BSA complex as determined by protein content, or the same amount of mock-treated BSA in PBS. After washing three times with PBS, wells were blocked with 1% (w/v) BSA. Wells were incubated for 2 h with rIL-6 diluted in PBS containing 50 μg/ml BSA. After washing three times with PBS containing 0.05% (v/v) Tween 20, 100 μl anti-IL-6 Ab was added at a dilution of 1/1000 in blocking buffer. Following three further washes in PBS-Tween, alkaline phosphatase-coupled rabbit anti-goat IgG second Ab was added at a dilution of 1/1000 in blocking buffer for 30 min. After five washes in PBS-Tween, phosphatase activity was detected by adding 100 μl/well *p*-nitrophenol phosphate solution. In some experiments, a competitive variant of the ELISA was used in which cytokine diluted in PBS was preincubated with soluble glycosaminoglycan for 30 min before the addition of 100-μl aliquots of this mixture to coated and blocked wells. Absorbances were read at 405 nm after an appropriate time of incubation at room temperature using a Dynatech MR5000 ELISA reader (Dynex Technologies, Billingshurst, West Sussex, U.K.). For each plate, readings were zeroed against replicate blank wells, which were blocked without prior coating, received no IL-6, and were incubated with first and second Abs, and developed as described above.

In some experiments, rhIL-6 was preincubated at lab temperature for 30 min in the presence and absence of 40 ng/well human srIL-6Rα polypeptide, in PBS containing 50 μg/ml BSA before addition to coated wells. These studies were extended by similar ELISA in which varying concentrations of srIL-6Rα were preincubated with and without 40 ng/well rhIL-6, and the resulting binding was detected with goat anti-IL-6Rα as first Ab, diluted in PBS containing 2% (w/v) dried skim milk.

ELISA of IL-6 binding to receptor polypeptides

The binding of rhIL-6 to surface-captured srIL-6Rα was investigated by coating ELISA plates overnight with the anti-IL-6Rα-specific mAb 17.6, 100 μl/well containing 600 ng of ammonium sulfate-purified Ig fraction in PBS. After washing, and blocking with PBS containing 1% dried skimmed milk powder for 30 min, wells were loaded with 5 ng/well srIL-6Rα in blocking solution for 90 min. rhIL-6, 10 ng/well, was preincubated for 30 min in the presence or absence of heparin in PBS containing 0.05% BSA. After washing, plates were developed with anti-IL-6 in blocking solution, and then with second Ab, as above.

To study the binding of rhIL-6 and srIL-6Rα to sgp130, ELISA plates were coated by incubation for 2 h at room temperature with 15 ng/well of sgp130, diluted in PBS containing 0.05% (w/v) BSA, and followed by three washes with PBS. Where subsequent binding was to be probed using polyclonal anti-IL-6, wells were blocked with PBS containing 1% BSA instead of 1% dried skimmed milk powder, which was otherwise used. rhIL-6, 80 ng/ml in PBS/0.05% BSA, was preincubated at room temperature in the presence or absence of heparin. After addition of 150 ng/ml srIL-6Rα, 100 μl of this mixture was added per well. Following overnight incubation at 5°C, wells were washed three times with PBS/1% BSA, and incubated for 30 min with 100 μl anti-IL-6 and diluted 1/1000 in PBS containing 1% dried skimmed milk powder. The remainder of the assay was performed as described above.

Proteolytic digestion of IL-6

Endoproteinase Lys-C was stored frozen as a stock solution, 0.1 μg/ml enzyme, in 20 mM Tris-HCl buffer, pH 8, containing 0.05% 2-ME. A 5-μl aliquot of enzyme diluted 30-fold in the same buffer was added to 50 μl containing 40 ng IL-6 of cytokine, also diluted in the same buffer, and incubated at 37°C. Digestions were conducted in the presence and absence of 50 μg/ml heparin, and the mixtures also contained 2 μg BSA as carrier protein from the commercial preparations of rhIL-6. Aliquots of the mixtures were removed at various time intervals, and immediately boiled. IL-6 was detected by immunoblotting using polyclonal anti-IL-6 and enhanced chemiluminescence (SuperSignal Kit; Pierce and Warriner, Chester, U.K.).

Bioassay of IL-6

BAF3-gp130 cells, murine cells stably transfected to express human gp130 (32), were maintained in RPMI 1640 medium containing 7% FCS, 1 mM glutamine, and 1 mM gentamicin. Cells were washed and transferred into a sulfate-free formulation of RPMI 1640 medium supplemented as above, but also in the case of some cells, with 10 mM sodium chlorate. After 1 wk, cells were plated out at a density of 3×10^4 per 100 μl well in the presence or absence of rIL-3, rIL-6, and srIL-6Rα. After 3 days at 37°C, high humidity, and 5% CO_2, cell viability was monitored by 4-h exposure to tetrazolium reagents (Promega, Southampton, U.K.).

Results

In ELISA experiments, binding of rhIL-6 to wells coated with a heparin-BSA complex was readily and routinely detectable, whereas binding to wells coated with mock-conjugated BSA was negligible (Fig. 1*a*). Similar binding was observed with rhIL-6 obtained from an alternative commercial source (Amersham Pharmacia Biotech; data not shown). The binding of rhIL-6 to the heparin-BSA complex is readily detectable with as little as 5 ng/well cytokine, and increases thereafter in a dose-dependent manner. Similar dose-responsive binding was observed when 1% skimmed milk powder was used as blocking agent in place of the routinely used BSA (data not shown). As may be seen in Fig. 1*b*, rmIL-6 also shows dose-dependent binding to the heparin-BSA complex. It is, however, notable that the resulting absorbances are lower than those obtained under identical conditions with rhIL-6, as seen in Fig. 1*a*. However, because study of the murine cytokine necessitates a change of the detecting Ab to one specific for rmIL-6, no rigorous quantitative comparison between the binding properties of rhIL-6 and rmIL-6 can be made on the basis of this data.

To confirm that the strong binding of rhIL-6 to the heparin-BSA complex is indeed due to the presence of the GAG chains, rhIL-6 was preincubated with increasing concentrations of heparin before addition to wells coated with either heparin-BSA or mock-treated BSA. As may be seen in Fig. 1*c*, soluble heparin competes effectively for binding to the heparin-BSA complex, with some 80% of inhibition attained in the presence of 50 μg/ml soluble heparin. In other experiments (see Figs. 2, 3, and 4), even higher percentages of inhibition of binding in the presence of soluble heparin were obtained. From the series of experiments represented by Fig. 1*c*, the concentration of heparin giving 50% inhibition (IC_{50}) was determined to be about 2 μg/ml.

The ELISA was used further in the competitive format to investigate the specificity of the interaction of rhIL-6 with heparin. As may be seen in Fig. 2, in addition to heparin, fucoidan, a sulfated fucose polymer, acts as a potent inhibitor of the binding of rhIL-6 to the immobilized heparin-BSA complex. Indeed, fucoidan was found to be a more potent inhibitor than heparin on a weight basis (data not shown). By contrast, the other GAGs studied employed are poor inhibitors. Analysis of variants using a general linear method with Tukey post hoc test showed that chondroitin sulfate A (chondroitin 4-sulfate) and HS from bovine kidney are significant inhibitors, whereas chondroitin sulfate B (dermatan sulfate) and chondroitin sulfate C (chondroitin 6-sulfate) provide no significant inhibition.

Because HSs are widely distributed in the tissues, but are also highly diverse in structure, we examined the ability of different representatives of this class of GAG to act as inhibitors of the binding of rhIL-6 to the heparin conjugate. As shown in Fig. 3, HSA and HSE both gave inhibitions of binding similar to that obtained with the commercial bovine kidney HS. Indeed, over a series of experiments, we were unable to demonstrate a reproducible difference in inhibitory activity. This is in direct contrast with

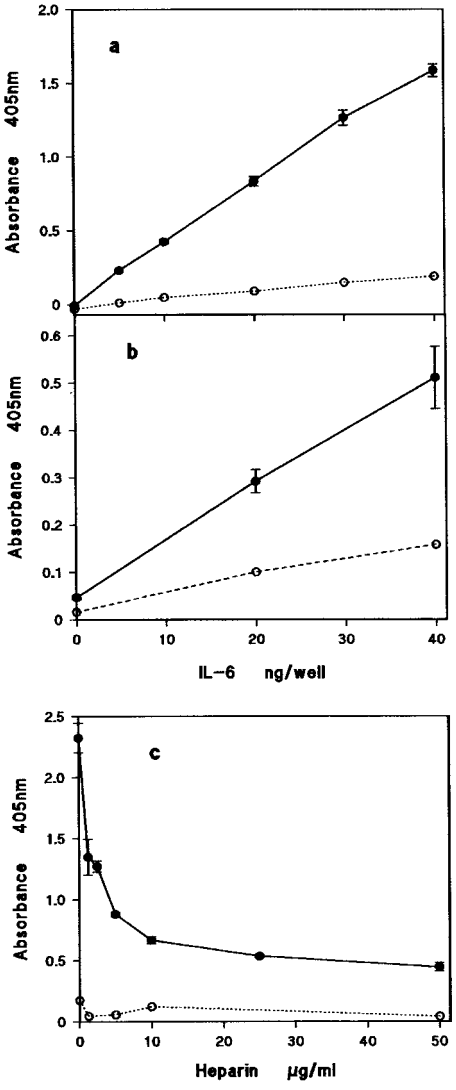

FIGURE 1. ELISA of the binding of rhIL-6 to immobilized heparin. *a*, Dose-dependent binding of rhIL-6. The results shown are typical of four independent experiments performed under the routine conditions described in *Materials and Methods*. *b*, Dose-dependent binding of rmIL-6. Performed as in *A*, except that the first Ab was 1/1000 diluted anti-murine IL-6. *c*, Competition with free heparin. rhIL-6, 20 ng/well, was preincubated with increasing concentrations of soluble heparin for 30 min before addition to the plate. The results shown are typical of three independent experiments performed under identical routine conditions. For all panels, each data point represents the mean of triplicate values. Solid symbols denoted binding to heparin-BSA complex; open symbols represent binding to mock-treated BSA. Errors bars indicate SEM, and are not shown for mock-treated BSA, in which this is less than symbol diameter.

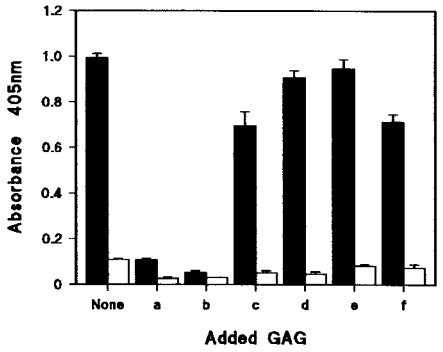

FIGURE 2. Specificity of binding of rhIL-6 to glycosaminoglycans. IL-6, 20 ng/well, was preincubated for 30 min on the absence (None) or presence of various GAGs: *a*, heparin; *b*, fucoidan; *c*, chondroitin sulfate A; *d*, chondroitin sulfate B; *e*, chondroitin sulfate C; *f*, HS from bovine kidney. All GAGs were present at 50 μg/ml, except fucoidan, 25 μg/ml. Solid columns represent wells coated with heparin-BSA conjugate, and open columns represent wells coated with mock-conjugated BSA. Each column represents the mean of quadruplicate wells, with error bars showing SEM. The results shown are representative of two independent experiments.

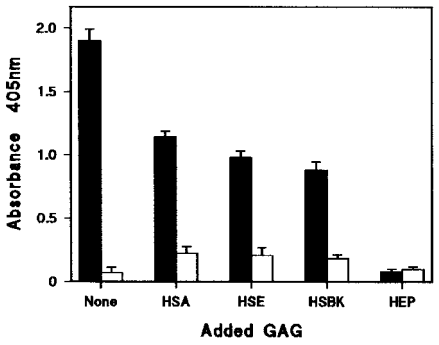

FIGURE 3. Comparison of different heparan sulfates as inhibitors of the binding of rhIL-6 to heparin. Incubations were conducted as for Fig. 2. HSA and HSE are respectively low and high sulfated fractions from porcine intestinal mucosa; HSBK, commercially obtained HS from bovine kidney; all HSs were used at 100 μg/ml. HEP, Heparin, 25 μg/ml. The results shown are representative of five similar experiments. Symbols and error bars, as for legend of Fig. 2, except that each point represents the mean of hextuplicate wells.

our previous results for the same preparations in our similar studies of the binding of rhIL-12 to heparin. In that work, the highly sulfated HSE was markedly stronger inhibitor of rhIL-12 binding than the other two HSs (29).

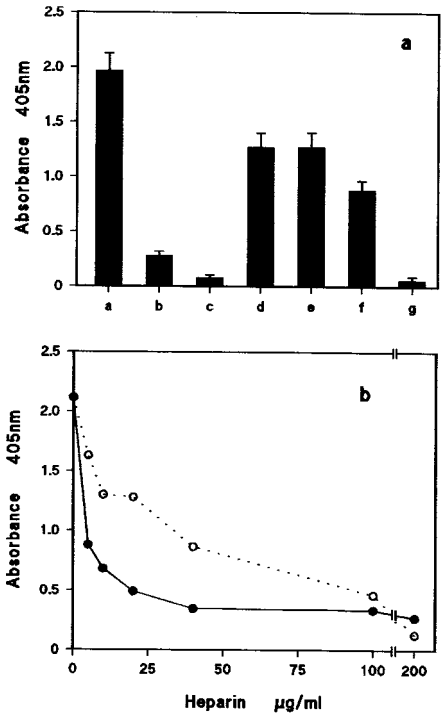

FIGURE 4. Inhibition of the heparin binding of IL-6 by chemically modified heparins. *a*, The soluble competitors were added at 125 μg/ml, unless otherwise stated: *a*, none; *b*, parent heparin added at 12.5 μg/ml; *c*, parent heparin; *d*, *N*-desulfated heparin; *e*, *N*-desulfated, *N*-reacetylated heparin; *f*, 6-*O*-desulfated heparin; and *g*, 2-*O*-desulfated heparin. *b*, Binding of IL-6 in the presence of increasing concentrations of parent heparin (solid symbols) and selectively 2-*O*-sulfated heparin (open symbols). The curves shown are typical of three independent experiments, and for the data shown the SEM was smaller than the diameter of the symbols. Background values for binding to wells coated with mock-treated BSA in place of the heparin-BSA complex have been subtracted. For both *a* and *b*, each result shown represents the mean of hextuplicate values. In *a*, error bars show ± SEM, but in *b*, this was smaller than the symbol diameter.

We also examined the ability of a series of chemically modified heparins derived from bovine lung heparin to compete for the binding of rhIL-6. Results from a representative experiment are shown in Fig. 4*a*. As may be seen, the parent unmodified bovine lung heparin, like the routinely employed porcine intestinal mucosal heparin, is a potent competitor of rhIL-6 binding. By contrast, *N*-desulfated bovine lung heparin is a very weak competitor. It produces less than 50% inhibition at the relatively high concentration employed, 125 μg/ml, compared with the parent heparin, which gives virtually complete inhibition of binding when used at the same concentration, and even at the lower concentration used (12.5 μg/ml) causes 80% inhibition. The further modification of the *N*-desulfated heparin by *N*-acetylation results does not alter this weak activity. Selective 6-*O*-desulfation of heparin similarly results in a weak inhibitor, although in this case the degree of inhibition is slightly higher. However, selective 2-*O*-sulfation results in a product that gives complete inhibition of binding. To compare fully the 2-*O*-sulfated product with its parent heparin, titration of inhibition of binding by these two preparations was performed. As shown in Fig. 4*b*, the parent bovine lung heparin is a potent competitor essentially indistinguishable from the heparin-obtained porcine intestinal mucosa (Fig. 1*b*). By comparison, the selectively 2-*O*-sulfation product, although achieving complete inhibition of binding at the high concentrations used, shows a much shallower inhibition curve. Thus, the IC_{50} for the 2-*O*-sulfated heparin is ~30 μg/ml, some 15-fold larger than that for the parent heparin.

To shed light on which surface regions might be involved in the interaction of IL-6 with heparin, ELISA studies were conducted in which the binding of the cytokine to the heparin-BSA complex was detected with anti-IL-6 mAbs rather than anti-IL-6 polyclonal Abs. For a series of five such mAbs, each recognizing a distinctive epitope (30), strong dose-dependent immunoreactivity was detected. A representative example of mAb 12 is shown in Fig. 5, but similar results were obtained with the other four mAbs. In only one case, mAb 15, was prolonged incubation of wells with enzyme substrate required to generate the high absorbances similar to those shown in Fig. 5. This prolonged incubation was consistent with the comparatively very low absorbances for this particular mAb obtained when it was used to detect the binding of IL-6 directly to ELISA well surfaces in the absence of the heparin-BSA complex (data not shown). We therefore concluded that in each case, the epitope of the mAb remained fully accessible after rhIL-6 had bound to heparin.

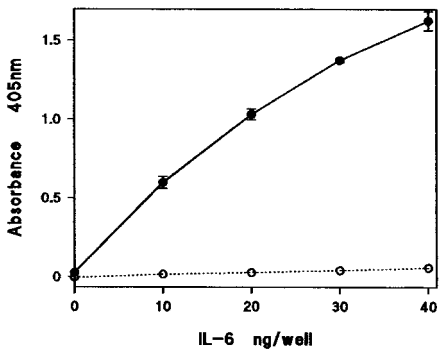

FIGURE 5. Binding of IL-6-specific mAb to heparin-bound IL-6. Symbols are as for Fig. 1, except each point represents the mean of quadruplicate wells.

Receptor-binding studies

An important question concerning the binding of a growth factor to heparin/HS is whether such binding might compete with the binding of the growth factor to its high affinity receptor polypeptides. This issue was addressed by performing binding experiments in which IL-6 was preincubated in the presence or absence of srIL-6Rα. As may be seen in Fig. 6*a*, srIL-6Rα causes no inhibition of IL-6 binding to heparin. Indeed, the binding of IL-6 is increased by the presence of srIL-6Rα at all concentrations studied. This stronger binding response occurs despite the possibility that the interaction with the soluble receptor might obscure Ab-binding determinants on the surface of IL-6, which are accessible to the detecting anti-IL-6 Ab when free IL-6 binds to heparin. It was found that srIL-6Rα alone binds negligibly to the heparin complex, as seen in Fig. 6*b*. However, when srIL-6Rα is preincubated with rhIL-6, strong binding of srIL-6Rα is seen. Thus, whereas free srIL-6Rα is not a heparin-binding polypeptide, the associated IL-6/srIL-6Rα dimer, like free IL-6, binds to heparin.

The three-way interaction between rhIL-6, sIL-6Rα, and heparin was further examined by capturing sIL-6Rα, via the IL-6Rα-specific mAb 17.6, to ELISA plate surfaces. As may be seen in Fig. 7, in the absence of heparin, high absorbances are obtained with IL-6, but only in the presence of srIL-6Rα. In the absence of the latter, background absorbances occur due to nonspecific binding of the detecting Abs to the wells. With increasing concentrations of soluble heparin, the specific binding of IL-6 to the immobilized sIL-6Rα is progressively inhibited, with maximal inhibition seen at 20 μg/ml heparin. This inhibition of IL-6 binding is, however, only partial. Over a series of three independent titration experiments, of which Fig. 7 shows a representative example, the average maximal inhibition was ~55%.

We next examined the effect of increasing heparin concentrations on the binding IL-6 and srIL-6Rα to sgp130-coated wells. As may be seen in Fig. 8, in the absence of heparin, IL-6 binds to these surfaces in the presence of sIL-6Rα, because only background levels of IL-6 binding occur in its absence. Preincubation of IL-6 and srIL-6Rα with heparin reduces the absorbance to these background levels. Maximal inhibition is seen with 10 μg/ml heparin, with near maximal inhibition observed with heparin concentrations as low as 2.5 μg/ml.

To demonstrate the binding of heparin to rhIL-6 by a method independent of the ELISA, we incubated rhIL-6 with the bacterial endoproteinase Lys-C. This enzyme cleaves at lysine residues which, having basic side chains, are likely to be involved in heparin binding. As may be seen in Fig. 9, which shows the results typical of five independent digestions, under the conditions employed in the absence of heparin there is rapid degradation of the single immunoreactive band that migrates with an estimated M_r of 21 kDa. This band is almost completely lost by 15 min, and is undetectable thereafter. However, in the presence of heparin, there is a much slower digestion of rhIL-6 that is progressive throughout the incubation period. Because the band at 1 h in the presence of heparin is considerably more intense than that seen at 15 min in the absence of heparin, we can conclude that heparin slows the rate of degradation of IL-6 more than 4-fold. We were unable to detect any fragments of rhIL-6 either in the presence or absence of heparin, indicating that cleavage of the polypeptide results in immediate loss of major epitopes. Simultaneous digestion at the same Lys-C concentration, of rhIL-10, a cytokine that fails to bind to the heparin-BSA complex in ELISA (unpublished data), shows only a slow rate of digestion over the same time course. However, the presence of heparin, far from protecting rhIL-10, increases the rate

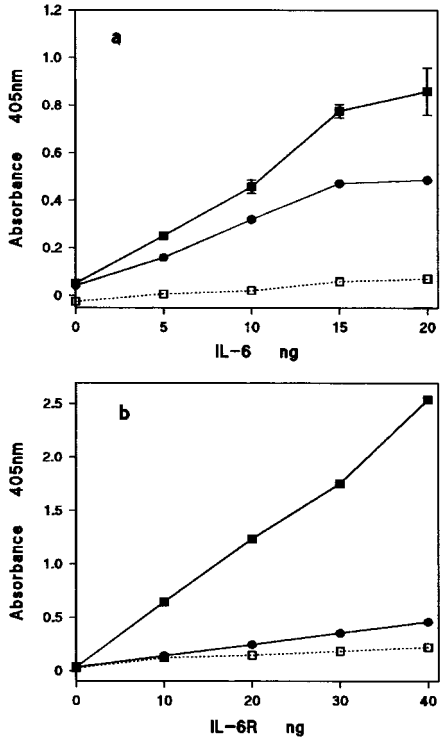

FIGURE 6. Binding of sIL-6Rα polypeptide and IL-6 to heparin. *a*, Binding of IL-6 to heparin in the presence and absence of srIL-6Rα. ●, IL-6 preincubated in PBS for 30 min in the absence of sIL-6Rα; ■,□, IL-6 preincubated in the presence of 40 ng/well sIL-6Rα. Solid symbols show binding to wells coated with heparin complex, and open symbols indicate binding to wells coated with mock-treated BSA. The binding of IL-6 preincubated in the absence of sIL-6Rα to wells coated with mock-treated BSA (data not shown) was superimposable on that obtained in its presence (□). *b*, sIL-6Rα binds to heparin only in the presence of IL-6. ●, srIL-6Rα preincubated in PBS for 30 min in the absence of rhIL-6; ■,□, srIL-6Rα preincubated in the presence of 40 ng/well IL-6. Solid and open symbols are as for *a*. The ELISA was developed with anti-IL-6Rα. The binding of srIL-6Rα to mock-treated BSA was indistinguishable whether it was incubated in the presence or absence of rhIL-6; hence, for clarity, the latter data are not shown. Throughout symbols are the means of triplicates and are shown ± SEM where this is larger than the symbol size. *a* and *B*, Each show a set of curves obtained in single representative experiments.

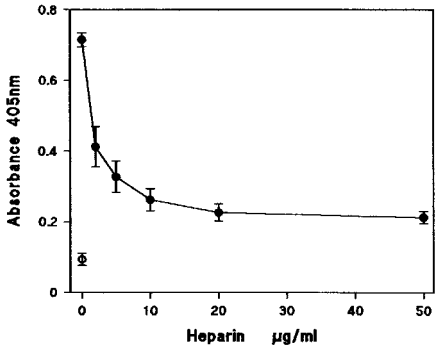

FIGURE 7. Effect of heparin on the binding of IL-6 to immobilized srIL-6Rα. The experiment was conducted as described in *Materials and Methods*, and the results are from a single experiment that is representative of two others. Solid symbols show wells incubated with rhIL-6 in the absence and presence of increasing concentrations of heparin. The open symbol incubates a well loaded with srIL-6Rα and then incubated in the absence of both rhIL-6 and heparin. All symbols indicated represent the means of hextuplicate wells, and error bars show ± SEM.

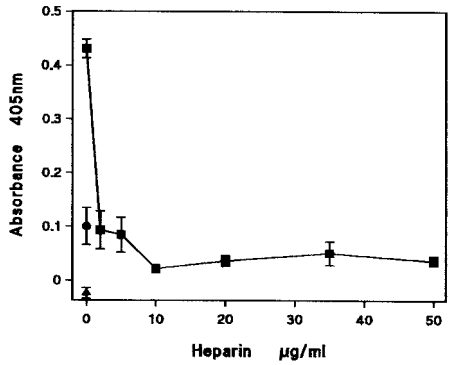

FIGURE 8. Effect of heparin on the binding of IL-6 and sIL-6Rα to immobilized sgp130. Wells were incubated with the following: ■, rhIL-6 and sIL-6Rα in the presence and absence of heparin; ●, srIL-6 in the absence of sIL-6Rα and heparin; ▲, srIL-6Rα in the absence of IL-6 and heparin. All wells were developed with polyclonal anti-IL-6 as first Ab. Each point represents the mean for hextuplicate wells, and is shown ± SEM. The results are for a single experiment that is representative of five.

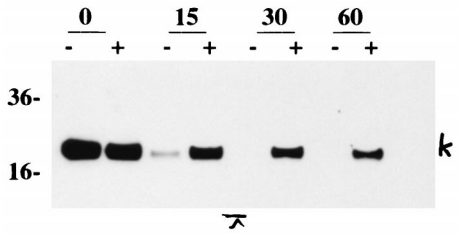

FIGURE 9. Protection of rhIL-6 from digestion with endoproteinase Lys-C. Numbering across the *top* indicates time of digestion at 37°C in min; + and −, indicate digestion in the presence and absence of 50 μg/ml heparin. The position and M_r in kDa of adjacent standards are indicated on the *left*.

of degradation, as seen by a greater loss of intensity detected at 20 kDa, and the appearance of a 16-kDa fragment that is not seen in the absence of heparin (data not shown). These findings with rhIL-10 indicate that the protection observed with rhIL-6 is specific, and not due to a generalized inhibition of Lys-C.

Effect of heparin on IL-6 bioactivity in B9 assay

Because chlorate, which interferes with HS biosynthesis by competitively inhibiting its sulfation, has been shown to block the biological activity of the heparin-binding cytokine FGF-2 (23), we investigated whether it might also affect the cellular activity of rhIL-6. When Ba/F3 cells expressing human gp130 are incubated in the presence of srIL-6Rα, there is a dose-dependent proliferation response to rIL-6 (data not shown). However, where the cells had been grown for 48 h in the presence of 10 mM sodium chlorate, the response to rhIL-6 is markedly less sensitive. This is particularly evident at low concentrations of rhIL-6, in the 5–10 ng/ml range. In the absence of chlorate, these concentrations provide over 50% of maximal proliferation, whereas in chlorate-treated cells, proliferation is minimal or undetectable. At the highest concentration of rhIL-6 employed, 150 ng/ml, the presence of chlorate caused over three independent experiments, a mean reduction of 71% in the otherwise optimal proliferative response that occurs in its absence. By comparison, the optimal proliferative response of these cells to IL-3 was reduced by a mean of 33%. Addition of soluble heparin had no reproducible effect on the proliferative response of Ba/F3-hgp130 cells to suboptimal concentrations of rhIL-6, whether the cells had been grown in the presence or absence of chlorate (data not shown).

Discussion

Despite their being relatively small, soluble glycoproteins, it is axiomatic that most cytokines act to a large extent locally within the tissues, i.e., in a paracrine rather than endocrine mode. An underlying mechanism of emerging importance is the binding of cytokines to GAGs, particularly heparin and HS. Among the ILs, work in our laboratory and elsewhere has shown that IL-2 (26, 27), IL-3 (33, 34), IL-4 (35), IL-5 (36), IL-7 (37), IL-8 (38), and IL-12 (29) all bind selectively to heparin and HS. We now report that IL-6 is also a heparin/HS-binding cytokine, demonstrating this in two independent ways. First, we show that in solution, heparin at low concentration is able to provide marked protection of rhIL-6 from digestion with a protease specific for Lys residues. Second, we have produced extensive data showing that rhIL-6 binds to immobilized heparin. This binding is dose dependent, and is displaceable with soluble heparin. The IC_{50} of 2 μg/ml for this competition compares with the equivalent values of 0.15, 5, and 0.1 μg/ml, which we previously obtained by the same ELISA method for the binding of FGF-2, IL-2, and IL-12 (26, 28). It is also similar to the value of 1 μg/ml we obtained in the same way for the well-established heparin-binding protein, antithrombin III (26). This indicates that the affinity of rhIL-6 for heparin is essentially similar to that for several other heparin-binding proteins.

We show that the interaction between rhIL-6 and heparin is specific, in that not all GAGs are, like heparin, effective competitors of the binding of the cytokine to the immobilized heparin complex. Fucoidan, a highly sulfated and branched chain polymer of L-fucose (39), isolated from marine algae, is a potent competitor, as is often observed with other heparin-polypeptide interactions. By contrast, the chondroitin sulfates show either weak (chondroitin sulfate A) or no inhibition (chondroitin sulfates B and C). This spectrum of activity within the chondroitin sulfate class of GAGs is unexpected because chondroitin sulfate B, dermatan sulfate, shares with heparin a high proportion of iduronate residues. Moreover, in our recent work on IL-12, the B form was established as the most active chondroitin sulfate inhibitor in that case (28). The HSs are a highly heterogeneous class of GAG and are widely distributed in the tissues, on cell surfaces, and in extracellular matrices. By contrast, heparin is a product of mast cells, stored in cytoplasmic granules, and released on degranulation. In the present study, HS from bovine kidney and porcine intestinal mucosa are shown to be inhibitors of rhIL-6 binding, albeit less active than soluble heparin. Little difference in activity was observed between the various HSs employed, and this contrasts with our similar studies on rhIL-12, in which the highly sulfated HSE was a markedly stronger inhibitor of binding than the lower sulfated HSA fraction isolated from the same source (28). Our use of a range of selectively modified heparin derivatives indicates that the various sulfate moieties, *N*-sulfates, 6-*O*-sulfates and 2-*O*-sulfates, all contribute partially to rhIL-6 binding, because their individual removal results in preparations in which heparin binding is reduced, but not abolished. Of the modified preparations, only the 2-*O*-desulfated heparin retains the ability to completely inhibit the binding of rhIL-6 to the complex, albeit with an IC_{50} some 15-fold higher than that of the unmodified parent heparin. This relatively dispensable role of 2-*O*-sulfates in rhIL-6 binding, contrasts with the essential role of 2-*O*-sulfates in the binding of FGF-2 to heparin (40). Thus, in detail, the specificity of the interaction of rhIL-6 with GAGs differs from that observed with other cytokines, including IL-12 and FGF-2.

We show, in this study, not only that rhIL-6 preincubated with srIL-6Rα binds to the heparin complex, but also that srIL-6Rα only binds to the heparin complex after preincubation with rhIL-6. This is clear evidence that the rhIL-6/srIL-6Rα dimer, a complex of physiological importance (20), like free rhIL-6, binds to heparin. However, we also find that, when srIL-6Rα is immobilized using mAb 17.6, which recognizes a conformation-dependent epitope yet to be delineated (44), heparin gives weak and partial inhibition of the subsequent binding of rhIL-6. Taken together, this suggests that the binding to srIL-6Rα may reduce but does not abolish the interaction between rhIL-6 and heparin. More striking inhibition of receptor binding is observed when immobilized sgp130 is used to capture rhIL-6/srIL-6Rα in the presence and absence of heparin. In this particular ELISA, rhIL-6 capture is seen to be dependent on the presence of srIL-6Rα, a finding entirely consistent with our understanding of the interaction between these three polypeptides (1, 18, 19). This specific binding of rhIL-6 is however strongly and completely inhibited by heparin, with an IC_{50} apparently of less than 2 μg/ml, the IC_{50} for the inhibition by soluble heparin of the binding of rhIL-6 to the heparin complex. Thus, overall, our data from receptor-binding experiments suggest that heparin may serve to reduce the availability of IL-6 to its receptor complex.

However, the physiological significance of this inhibition remains to be established. It is most likely that the affinity of the IL-6/IL-6Rα dimer for gp130, $K_d \cong 10$ pM (42), considerably exceeds that for heparin/HS, and thus the GAG would be displaced, provided favorable concentrations of the receptor polypeptide exist on the cell surface. Moreover, there is considerable microsequence heterogeneity within the heparin/HS GAG family. Because our findings suggest a degree of structural specificity in interaction between IL-6 and GAGs, the nature of the available heparin/HS will be important too.

To begin examining the possible effects of heparin and HS on the activity of IL-6, we studied murine Ba/F3 cells stably transfected to express human gp130. Growth of these cells in low sulfate media in the presence of chlorate reduces their responsiveness to IL-6. Such treatment blocks the biological activity of the heparin/HS-dependent cytokine FGF-2 (23), and substantially lowers the efficiency of infection of lymphoid cells by herpes simplex virus type 1, an HS-dependent process (43). These data imply that sulfated GAGs play a positive role in IL-6 signaling in these particular cells.

The IL-6 mAbs used in this study all potently inhibit the bioactivity of rhIL-6 (30), indicating that their epitopes either contribute to, or lie in close proximity with, sites on the surface of IL-6, which are important for the engagement of the receptor polypeptides. All five IL-6-specific mAbs used in this study still bind to the cytokine captured on the heparin-BSA complex. This establishes that each of their respective epitopes remains available after heparin binding. In the case of three of these mAbs, the epitopes have been mapped by site-directed mutagenesis. Thus, the epitope of mAb 8 encompasses residue Phe^{79} in the AB loop region as well as five residues at the C terminus, Arg^{180}-Met^{185} (41). (All residue numbering is according to Xu et al. (16).) These contribute to site I, a binding site for the receptor polypeptide IL-6Rα (44). The epitope for mAb 12 involves residues Tyr^{32} and Gly^{36} of helix A, and Ser^{119} and Val^{122} of helix C (44), all critical residues in site II, one of two binding sites for gp130 (44, 45). The mAb 16 epitope is abolished by mutation of Glu^{160} and Thr^{163}, which occur at the beginning of the D helix and contribute to site III, the second gp130 binding site. Our evidence for blockade of gp130 binding at sites II and/or III contrasts with our observations that the epitopes of mAbs 12 and 16 remain available after heparin binding. This suggests that in respect to heparin binding, the epitopes of these mAbs and the gp130 binding sites may not correspond exactly.

Several hypotheses may be advanced to explain the effects of heparin on IL-6R polypeptide binding. One is that there are multiple heparin binding sites on IL-6 that may be employed preferentially under particular experimental conditions. However, an attractive hypothesis arises from consideration of the possible heparin binding site(s) on the surface of IL-6. Heparin binding sites typically involve four to six basic amino acids, Arg or Lys (reviewed by Ref. 46), either as a single cluster, or as clusters spaced ~20Å apart, so as to fit the periodicity of sulfate groups along a single face of the helical GAG chain (47). Inspection of the high resolution structures of rhIL-6 (15, 16) reveals a face comprising the A and D helices rich in basic residues and lacking acidic residues that would interfere with heparin binding through charge repulsion. As may be seen in Fig. 10, relatively central in this face is a cluster of four basic residues: Arg ^{41} and Lys ^{42} in the A helix, and Arg^{169} and Lys^{172} from the D helix. ~20Å away there are further basic residues, Arg^{180} and Arg^{183} in the D helix, and Lys^{28} and Arg^{31} in the A helix. It is entirely plausible that a heparin/HS chain could bind to these residues and thus lie across this face, parallel to the A and D helices. Alignment of 14 mammalian IL-6 amino acid sequences (48) shows that of these eight basic residues, four (Arg^{41}, Lys^{42}, Arg^{180}, and Arg^{183}) are completely conserved. A further two residues, Arg^{169} and Arg^{31}, show a high degree of conservation.

It may be further hypothesized that if site I or site II were already occupied by their respective polypeptide ligands, accessibility of some of these basic residues would be restricted. For instance, the binding of IL-6Rα at site I would block the residues at the carboxyl terminus. Such blocking would leave other basic residues on this face available, and therefore would alter but not abolish binding. Alternatively, if heparin binds first across this face, then binding of the receptors may be either restricted (IL-6Rα at site I) or prevented (gp130 at sites II and III). In this way, all of our

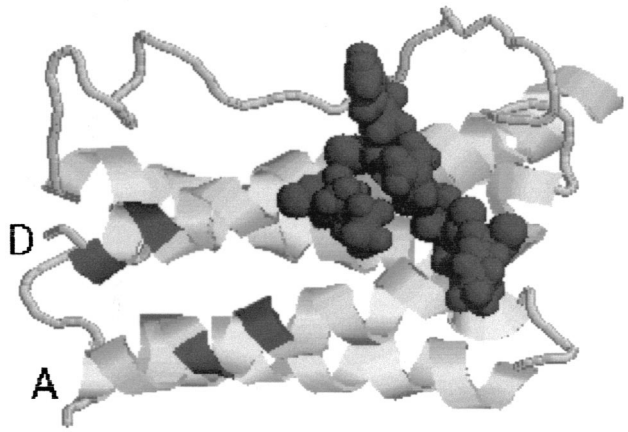

FIGURE 10. Model of IL-6 displaying basic residues that constitute a putative heparin binding site. The model was generated with RASMOL using a cartoon backbone format to display the α-helices and interconnecting loops. The amino terminus of the A helix (residues 21–47) and carboxyl terminus of the D helix (residues 156–185) are indicated by the single letters. In this view, the AB loop (48–80) lies across the top of the structure, the B helix (81–105) lies behind the D helix, and the C helix (109–131) lies behind the A helix. The four basic residue cluster is highlighted in dark spacefill, and comprises Lys^{172} (*left*), Arg^{169} (*top*), Arg^{41} (*center*), and Lys^{42} (*bottom right*). Arg^{180} and Lys^{183} in the D helix, and Lys^{28} and Arg^{31} in the A helix are highlighted as dark ribbon. Residues numbering is according to Xu et al. (16).

observations on soluble receptor polypeptide and heparin binding may be accounted for.

Irrespective of how heparin/HS affects IL-6R engagement, it is likely that other consequences of cytokine-GAG interactions will apply in the case of IL-6. These include the restricted diffusion of such small, soluble glycoproteins away from tissue microenvironments of secretion (reviewed in Ref. 49). This therefore provides a mechanism for achieving a paracrine mode of activity. An important corollary of this is that the levels of IL-6 measured in the serum in pathophysiological states may be poorly representative of the activity of IL-6 in particular tissue compartments.

Through the action of extracellular digestive enzymes, either proteases cleaving the core polypeptide of a proteoglycan, or heparinase cleaving the GAG chains, a bound cytokine may be released still bound to a proteoglycan fragment. It has already been demonstrated that IL-6 circulates as a series of complexes that cannot be accounted for entirely by the association of IL-6 with soluble receptor polypeptides and autoantibodies (50). The possible contribution of proteoglycan fragments to these transport complexes now warrants investigation. Likewise, our present findings indicate that a detailed investigation of the effects of heparin/HS GAGs on the physiological and pathological activities of IL-6 is now required. In this context, it is highly significant that the synthetic polysulfated napthyl urea suramin, an antitumor and anticachexia agent that acts as a heparin mimetic in several biological systems, not only inhibits the biological activities of IL-6, but also inhibits the binding of IL-6 to its cell surface receptors (51, 52).

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CORRECTIONS

Rosemary S. Mummery and Christopher C. Rider. Characterization of the Heparin-Binding Properties of IL-6. *The Journal of Immunology*, 2000, 165: 5671–5679.

In *Results*, Figure 9 was incorrectly published with the hand-drawn alignment arrows along the right and bottom edge of the figure. The correct figure is shown below.

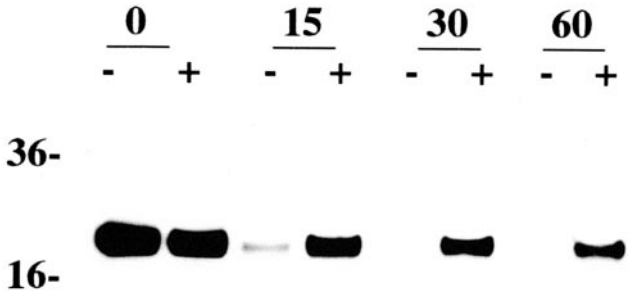

FIGURE 9. Protection of rhIL-6 from digestion with endoproteinase Lys-C. Numbering across the *top* indicates time of digestion at 37°C in min; + and −, indicate digestion in the presence and absence of 50 μg/ml heparin. The position and M_r in kDa of adjacent standards are indicated on the *left*.

Mitsuhiro Yamada, Hiroshi Kubo, Seiichi Kobayashi, Kota Ishizawa, Muneo Numasaki, Shinsaku Ueda, Takashi Suzuki, and Hidetada Sasaki. Bone Marrow-Derived Progenitor Cells Are Important for Lung Repair after Lipopolysaccharide-Induced Lung Injury. *The Journal of Immunology*, 2004, 172: 1266–1272.

In *Results*, panel *A* of Figure 5 was inadvertently duplicated in panels *D* and *E*. The correct figure is shown below.

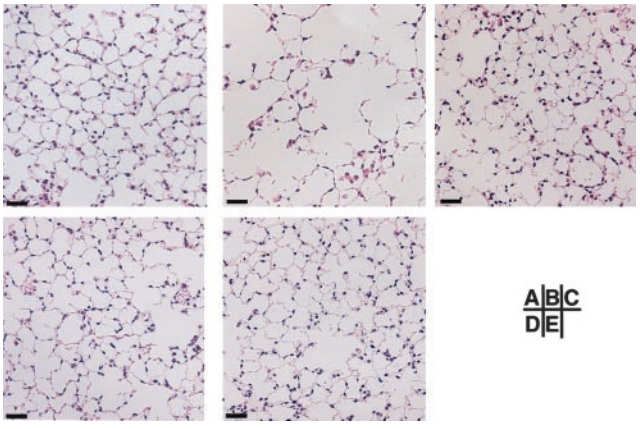

FIGURE 5. LPS induces emphysema-like changes in mice when BMPCs were suppressed by irradiation. *A–E*, Lung H&E-stained sections of mice 1 wk following: *A*, intranasal PBS; *B*, intranasal LPS in irradiated (5 Gy) mice; *C*, intranasal LPS in irradiated and reconstituted mice; *D*, intranasal PBS in irradiated (5 Gy) mice; and *E*, intranasal LPS. Scale bars, 50 μm.

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Andrew I. Webb, Natalie A. Borg, Michelle A. Dunstone, Lars Kjer-Nielsen, Travis Beddoe, James McCluskey, Francis R. Carbone, Stephen P. Bottomley, Marie-Isabel Aguilar, Anthony W. Purcell, and Jamie Rossjohn. The Structure of H-2K^{b} and K^{bm8} Complexed to a Herpes Simplex Virus Determinant: Evidence for a Conformational Switch That Governs T Cell Repertoire Selection and Viral Resistance. *The Journal of Immunology,* 2004, 173: 402–409.

In *Results,* Figure 2 was incorrectly printed in grayscale. The correct color figure is shown below.

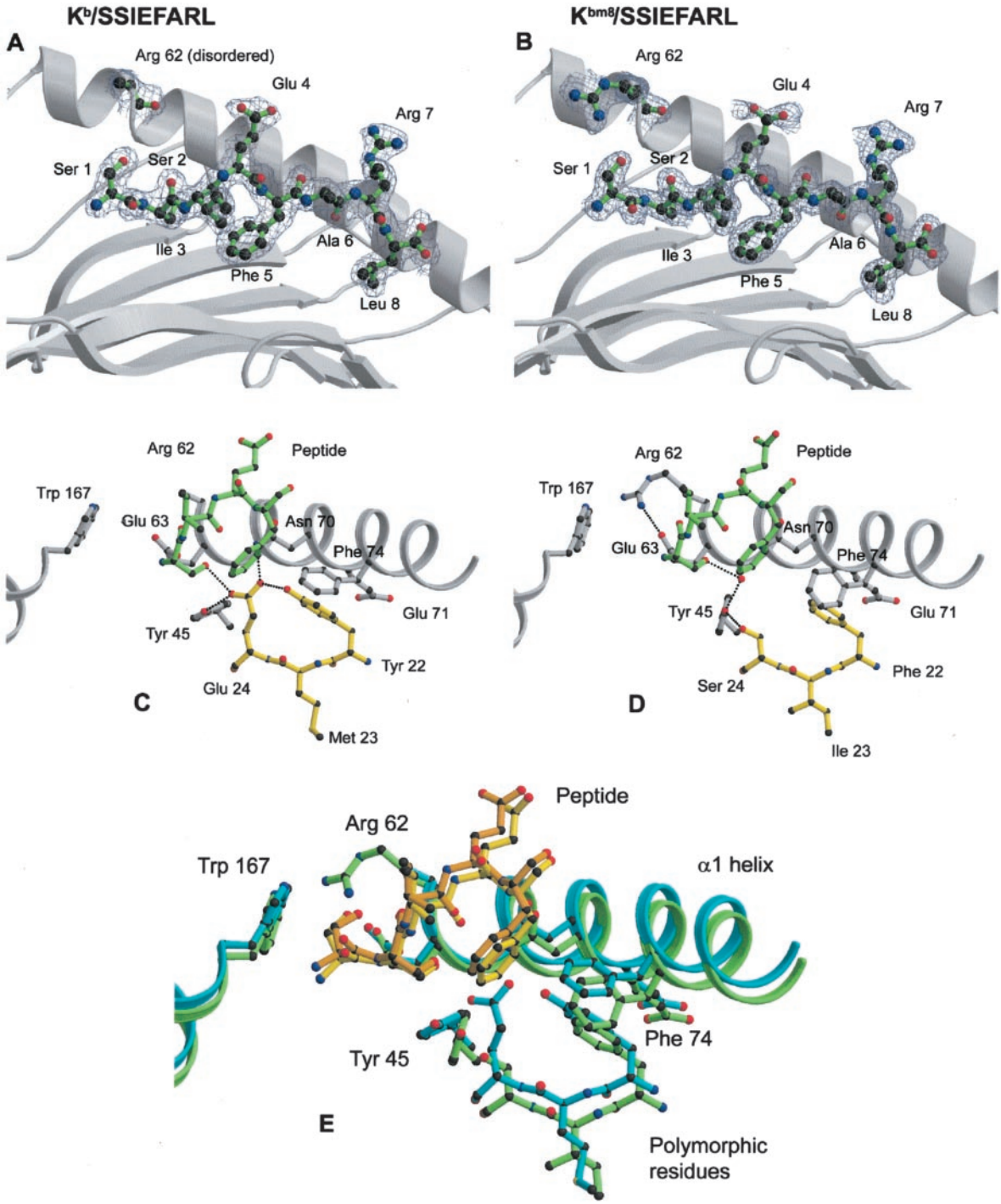

FIGURE 2. Cut-away view of the Ag-binding clefts of K^{b} (*A*) and K^{bm8} (*B*) bound to the SSIEFARL. The 2.0- and 1.8-Å electron density omit maps of SSIEFARL complexed to the respective H-2K molecules are also indicated. Very similar conformations of the peptide were observed, highlighting the exposed Ser^{1}, Glu^{4}, Ala^{6}, and Arg^{7} residues. Analysis of H-bond and van der Waals contacts contributed to by polymorphic amino acids in K^{b} (*C*) and K^{bm8} (*D*) are shown in the same orientation as the views in *A* and *B*, respectively. These representations were also superimposed (*E*) to highlight the mobility of the Arg^{62} residue and the rigid-body shift in the α_1 helix (residues 62–73). The K^{b} hc is shown in cyan, whereas K^{bm8} is shown in green. The peptide ligands are shown in orange and yellow for K^{b} and K^{bm8}, respectively.