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Characterization of an Interleukin 6 Cytokine Family Antagonist Protein from a Marine Sponge, *Callyspongia sp.**

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An inhibitor of IL-6 binding to the human hepatoma line HepG2 and myeloma cell line U266 was identified in a saline extract of the marine sponge, *Callyspongia sp.* Functional activity, measured through the increase in haptoglobin production by HepG2 cells stimulated with IL-6, could be strongly inhibited by the extract. Similarly, IL-6-induced production of IgM by the B cell line SKW6.4 was substantially reduced. In neither cell line was there evidence of toxicity produced by the extract. Other sponges of the *Callyspongia* species were found to contain analogous activity. The activity was destroyed by trypsin treatment or boiling of the extract, suggesting that the inhibition is due to a protein. When the binding of IL-6 to its receptor complex was dissected *in vitro*, inhibition of binding of IL-6 to soluble receptor by the extract was not detected, but binding of the IL-6-sIL-6R complex to soluble gp130 was inhibited in a dose-dependent fashion. This was borne out in cellular assays since the extract inhibited activation of HepG2 cells stimulated with oncostatin M or leukemia inhibitory factor, cytokines which also use gp130 for signal transduction. These results suggest that the *Callyspongia* extract contains a protein which blocks the interaction of the IL-6 family of cytokines with their signal transduction moiety, gp130. Elucidation of the structure and mode of action of such a protein would be helpful in designing gp130 antagonists to inhibit the functions of this cytokine family, overproduction of which has been associated with cancer and pathologies of autoimmune disease and AIDS.

IL-6 and related cytokines such as OSM,¹ LIF, IL-11, and

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¹ The abbreviations used are: OSM, oncostatin M; IL, interleukin; LIF, leukemia inhibitory factor; (s)IL-6R, (soluble) IL-6 receptor; sgp130, soluble gp130; PBS-A, Dulbecco's phosphate-buffered saline, pH 7.2, 0.1% sodium azide; BSA, bovine serum albumin; PMPs, paramagnetic particles; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

ciliary neurotrophic factor play important roles in the immune system, hematopoiesis, the nervous system, and acute phase reactions (1). These cytokines are structurally similar, having a predicted four α -helical bundle structure, and show overlapping spectra of individual activities (1–4). Their biological activities are mediated through initial low affinity binding to cell surface receptors, which are specific for their respective ligands yet structurally related (1, 2, 5–7). In addition, they share a receptor subunit, the transmembrane glycoprotein gp130, which is responsible for initiating signal transduction (3, 5, 8). Once the receptor complex is fully formed, the ligand binding affinity is much increased (5). For example, the K_d of IL-6 for receptor alone is 1 nM but increases to 45 pM when gp130 is engaged (9). The combination of expression of specific cytokine receptors by particular cell types with a sharing of the ubiquitously expressed gp130 (10) to enable signal transduction provides some explanation for the observed functional overlap or biological redundancy between members of this cytokine family. The sharing of gp130 thus provides a useful target by which to inhibit the function of this cytokine family (8), members of which have been implicated in the pathology of several diseases, such as AIDS, cancer, and autoimmune diseases (1).

In this paper we describe the activity of a protein discovered in an extract of the marine sponge *Callyspongia*, which inhibits the formation of receptor complexes involving gp130, consequently inhibiting the bioactivity of IL-6 and other members of this cytokine family.

EXPERIMENTAL PROCEDURES

Cells and Reagents—U266 (human myeloma), HepG2 (human hepatoma), and SKW6.4 cells (human Epstein Barr virus-transformed B cell) were all obtained from ATCC (Rockville, MD). U266 and SKW6.4 cells were maintained in RPMI 1640 with 10% fetal bovine serum and 1% each of penicillin, L-glutamine, and streptomycin. HepG2 cells were maintained in Earle's minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin, streptomycin, L-glutamine, non-essential amino acids, and sodium pyruvate (Life Technologies, Inc.). Human recombinant cytokines, sIL-6R and sgp130, were purchased from R & D Systems.

Sponge Collection, Identification, and Preparation of Extract—Sponges collected by scuba were identified by microscopic evaluation of spicules and fiber architecture. The three sponges used here were species of *Callyspongia* (Porifera, Desmospongiae, Haplosclerida, Callyspongiidae). *C. armigera* (sample 3-XII-92-2-004) and *C. vaginalis* (sample 3-XII-92-4-002) (11) were collected at 100-foot and 20-foot depths, respectively, off south Eleuthera, Bahamas. The third, designated *Callyspongia sp.* (sample 16-VIII-90-1-003), was collected at 50-foot depth off Ponta Santa Cruz, Madeira, and is most closely related to *C. simplex* (12). The major difference is in the size/morphology of the oxeas; however, since this can be influenced by environmental factors, the sponge may be conspecific with *C. simplex*. Taxonomic vouchers for the three sponges have been deposited in the Harbor Branch Oceanographic Museum (catalog numbers 003:00923, 003:00924, and 003:00922, respectively). Samples were stored at -20°C until extraction. Except where stated, all results pertain to experiments with *Callyspongia sp.* extract. About 10 g wet weight of frozen sponge was used to prepare extracts. With mortar and pestle set in a dry ice bath, the sponge was powdered, 20 ml of ice-cold PBS-A was added, the preparation was freeze-thawed 3 times, and centrifuged at $20,000 \times g$ for 30 min. The pellet was resuspended in 10 ml of buffer and centrifuged again. Supernatants were pooled and frozen at -20°C . Total protein concentration of the mixture present in the extract was estimated with the BCA assay (Pierce Chemical Co.), using a BSA standard.

Bioassays—Functional activity of IL-6 was assessed through the increase in haptoglobin secretion by HepG2 cells and induction of IgM

secretion by SKW6.4 cells. The HepG2 assay was adapted from methods already described (13): HepG2 cells grown to confluency in 96-well plates were treated for 4 h with 10 ng/ml IL-6 in 100 μ l of their normal growth medium, with or without extract, in quadruplicate. Controls were included where no IL-6 was added. After a further 48 h of culture in normal medium, the medium was removed for assay of haptoglobin. The assay using SKW6.4 cells was set up in an analogous fashion, using cells in log phase growth and 10,000 cells/200 μ l of medium; again, cells were treated with cytokine and inhibitor for 4 h and after a further 72-h incubation, the medium was removed for IgM assay. Cell proliferation was measured (MTT) using a CellTiter96 assay kit (Promega).

Immunoassays—Haptoglobin was measured by radioimmunoassay as described previously (13). The working range was 0.1–10 ng/ml haptoglobin. IgM was measured using a “sandwich” enzyme-linked immunosorbent assay method. Goat anti-human IgM (Boehringer Mannheim Biochemicals) was bound to 96-well plates as first antibody, samples or standard IgM was incubated in the wells for 2 h; the same antibody conjugated to alkaline phosphatase was used to detect bound IgM, and the working range was 50–500 ng/ml.

Binding of 125 I-IL-6 to Cells—HepG2 cells were plated into 35-mm Petri dishes and grown to almost 100% confluency (2×10^6 cells). All procedures were carried out with 1 ml of normal cell growth medium. After 2 washes, binding of 10 pM 125 I-IL-6 (approximately 100 μ Ci/ μ g; DuPont NEN) was carried out at 4 $^{\circ}$ C for 4 h. Cells were washed 3 times, then solubilized at 37 $^{\circ}$ C in 1 ml of 1 M NaOH with 0.1% SDS before counting. For U266 cells, a suspension cell line, binding (10 pM 125 I-IL-6) to 10^4 cells in a 250- μ l volume was compared at 4 $^{\circ}$ C for 4 h or at 20 $^{\circ}$ C for 2 h. Unbound IL-6 was removed by rapid filtration through Whatman GF/C glass fiber filters presoaked in 2% nonfat dried milk solution. The filters were washed twice in cold 0.9% NaCl and assessed for radioactivity. Results were found to be very similar for both conditions, and the 2-h binding method was selected for all further U266 experiments. In typical experiments, K_d values for HepG2 and U266 cells were determined as 119 pM and 70 pM respectively, similar to values already published by others for the high affinity site for IL-6 binding on U266 cells (9). In contrast to this study, however, Scatchard analysis demonstrated no significant numbers of low affinity sites on our clone of U266 cells (data not shown).

Binding Conditions for Forming Complexes of 125 I-IL-6, sIL-6R, and sgp130—A constant input (7 pM, 20,000 cpm) of 125 I-IL-6 was mixed with varying amounts of sIL-6R from 1 nM to 2 pM in 200 μ l of PBS-A containing 1% BSA. Binding of IL-6 to sIL-6R (2 h/20 $^{\circ}$ C) was assessed by immunoprecipitation using an excess of non-neutralizing mouse anti-IL-6R (Biosource) bound to rabbit anti-mouse IgG coupled to PMPs (Ciba Corning Diagnostics). PMPs were washed 3 times with 0.1 M Tris-HCl, pH 7, + 0.1% Tween 20 before counting. Background binding, assessed by omitting sIL-6R, was around 700 cpm. A concentration of 125 pM sIL-6R gave total binding around 2,000 cpm; this was then used to measure the increase in IL-6 binding when sgp130 was included. Through a similar titration method, 540 pM sgp130 was found to increase total binding to about 3,000 cpm. These conditions were used to examine the mechanism of action of inhibitors of IL-6 function. In some experiments, when sgp130 was present, receptor-ligand complexes were pulled down using polyclonal goat anti-gp130 (R & D Systems) on rabbit anti-goat IgG PMPs (Perceptive Diagnostics); this method gave a similar pattern of results to experiments with anti-IL-6R PMPs.

RESULTS AND DISCUSSION

Inhibition of Binding—The *Callyspongia* sp. extract (estimated total protein 0.5 mg/ml) inhibited 125 I-IL-6 binding to HepG2 cells in a dose-dependent manner as illustrated in Fig. 1, giving 50% inhibition at 1:6,200. This extract, and those of the two other *Callyspongia* organisms tested, showed similar inhibition of IL-6 binding in U266 binding (IC_{50} of 1:1,600 for *C. armigera*, and 1:3,200 for *C. vaginalis*, compared with 1:5,900 for *Callyspongia* sp.). The extract had no effect on the binding of 125 I-IL-1 to its receptor on ELA.6.1 cells or of 125 I-IL-4 to the IL-4R of Raji cells (data not shown), indicating ligand/receptor specificity.

Activity in Functional Assays for IL-6—HepG2 cells respond to cytokines such as IL-6 and IL-1 by increased production of certain acute-phase proteins, e.g. haptoglobin. IL-1 has been reported to up-regulate haptoglobin production by HepG2 cells less effectively than IL-6 (14), which we also observed; however, maximum up-regulation of haptoglobin by IL-6 or IL-1

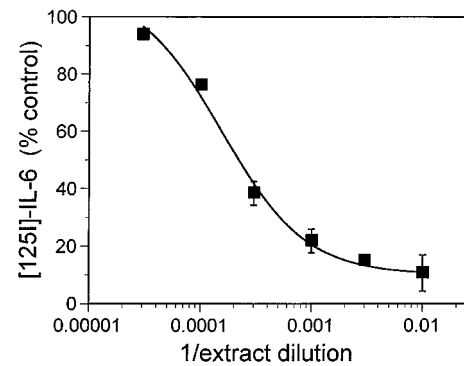


FIG. 1. Inhibition of 125 I-IL-6 binding to HepG2 cells by dilutions of the sponge extract. Input was 30,000 cpm, maximum binding (no extract) was 2,300 cpm; results are expressed as percent of total binding. Each point was carried out in triplicate, and the error bars represent the S.D.

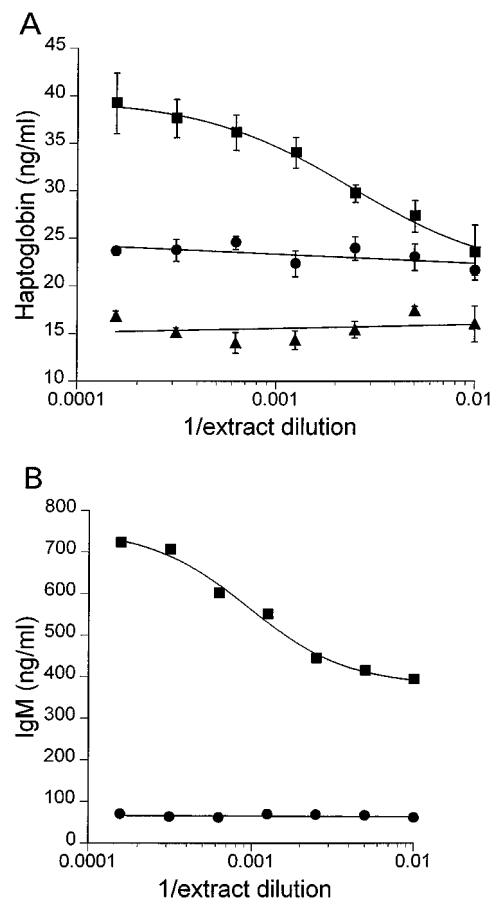


FIG. 2. Inhibition of cellular responses to IL-6 by sponge extract. A, HepG2 cells were incubated in the presence of extract dilutions and 10 ng/ml IL-6 (■), IL-1 (●), or no cytokine (▲). Haptoglobin produced in the absence of sponge extract was 38.9 ± 1.2 ng/ml (+IL-6), 22.5 ± 1.3 ng/ml (+IL-1), and 17.8 ± 1.5 ng/ml (no cytokine). Each point is the mean of 4 replicates; error bars represent S.D. B, SKW6.4 cells were incubated in the presence of extract dilutions and 10 ng/ml IL-6 (■) or no cytokine (●); each point is the mean of 4 replicates, and the S.D. was always less than 12%. Mean IgM produced in the absence of extract was 824 ng/ml (+IL-6) and 62 ng/ml (no IL-6).

was found to occur at about 10 ng/ml for both cytokines (data not shown). To discover whether inhibition of binding by the extract was due to the presence of an agonist or an antagonist for IL-6, HepG2 cells were incubated for 4 h with extract dilutions plus 10 ng/ml IL-6 or IL-1, or neither. After a further 48 h, haptoglobin was measured (Fig. 2A). The extract inhibited IL-6-stimulated up-regulation of haptoglobin in a dose-

pendent fashion, with an IC_{50} of 1:430, but did not affect the basal production of haptoglobin when present without IL-6, indicating that the extract was neither cytotoxic to HepG2 cells nor a general protein synthesis inhibitor. As expected, IL-1 increased the level of haptoglobin secretion to a lesser extent than IL-6; however, this increase was not responsive to the presence of extract. In other experiments (Fig. 2B), the extract inhibited the IL-6-induced production of IgM by SKW6.4 cells, with an IC_{50} of 1:1,050. These cells were also tested using the MTT assay, and the extract did not change the rate of cell proliferation or affect their viability.

The results in functional assays indicate that the inhibition of IL-6 binding to cells was due to an antagonist present in the extract. Using ^{125}I -IL-6 binding to U266 cells to follow activity, it was shown that the inhibition was completely lost after heating the extract for 5 min at 100 °C and after treatment at 37 °C with 1% trypsin for 10 min. Activity was >80% retained by ultrafiltration membranes (Amicon) with M_r retention of 10,000 and by cellulose dialysis membranes of 12,000–14,000 M_r cut-off. Activity could be concentrated by precipitation with 50% saturated ammonium sulfate solution, with a 40% recovery of activity in the precipitate. In addition, the heated extract was checked in the HepG2 functional assay and found to be completely inactive. These data indicated that the activity was due to a protein present in the sponge extract.

HepG2 cells were preincubated for 1 h at 4 °C with extract at 1:1000, which was then removed, and the cells were washed. Subsequent binding of ^{125}I -IL-6 to these cells was inhibited by an amount ($63.7 \pm 0.9\%$) similar to that achieved by a 4-h co-incubation with extract (Fig. 1). Furthermore, the inhibitory activity contained in the extract taken off the cells was found to be progressively reduced in subsequent 1-h incubations at 4 °C with fresh HepG2 cells, from $63.7 \pm 0.9\%$ to $59.1 \pm 0.9\%$ after the first absorption, to $46.4 \pm 2.8\%$ after a second. This experiment suggested that the inhibitor bound to a component of the complex on the cell surface rather than to IL-6 itself.

Dissection of the Receptor Complex—In order to find out which part of the receptor complex was being inhibited by the extract, the soluble forms of the IL-6R and gp130 were used to set up a ^{125}I -IL-6 binding assay. We used anti-IL-6R on PMPs to immunoprecipitate complexes of IL-6-sIL-6R or of IL-6-sIL-6R-sgp130 formed in the presence or absence of extract (Fig. 3A). No change in binding of IL-6 to sIL-6R could be detected in the presence of extract; however, it abolished the enhancement of IL-6 binding conferred by sgp130 in a dose-dependent manner. Precipitation of complexes containing sgp130 with anti-gp130 PMPs also demonstrated dose-dependent inhibition of binding by extract (Fig. 3B).

Activity in Functional Assays with Other IL-6 Cytokine Family Members—Since OSM and LIF have both been demonstrated to increase acute-phase protein production by hepatoma cells and also utilize gp130 to transduce signals (8), we examined whether the sponge extract would also inhibit these cytokines in the HepG2 assay. We found that a 4 h "pulse" of OSM and LIF up-regulated haptoglobin production by HepG2 cells in extent and dose range closely analogous to IL-6, so the effect of the extract on the stimulation of HepG2 cells by IL-6, OSM, or LIF, all at 5 ng/ml, was compared (Fig. 4). All three cytokines were inhibited by the extract, but the maximum degree of inhibition of OSM and LIF achieved in these experiments (around 35%) was lower than for IL-6 (nearly 90%). IL-6 requires a disulfide-linked homodimerization of two gp130 molecules to elicit signal transduction (15), whereas the current evidence regarding OSM and LIF function indicates that only one gp130 molecule is included in the receptor complex (1). This suggests that, even if the site of interaction of the various

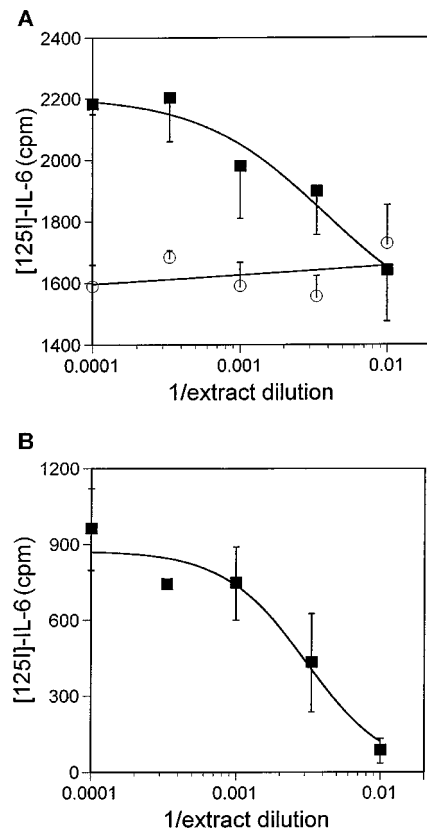


FIG. 3. **Immunoprecipitation of IL-6-sIL-6R-gp130 complexes.** A, ^{125}I -IL-6 was incubated with sIL-6R with (■) or without (○) sgp130, in the presence of extract dilutions. Immune complexes were pulled out of solution with anti-IL-6R on PMPs. Nonspecific binding was determined by running parallel determinations excluding sIL-6R, for each dilution of extract, and this was subtracted from the results. B, ^{125}I -IL-6 was incubated with sIL-6R and sgp130 (■), in the presence of extract dilutions. Immune complexes were pulled out of solution with anti-gp130 on PMPs. Nonspecific binding was determined by running parallel determinations using PMPs with no anti-gp130 antibody, for each dilution of extract, and this was subtracted from the results. For A and B, points were run in triplicate, and the error bars represent S.D.

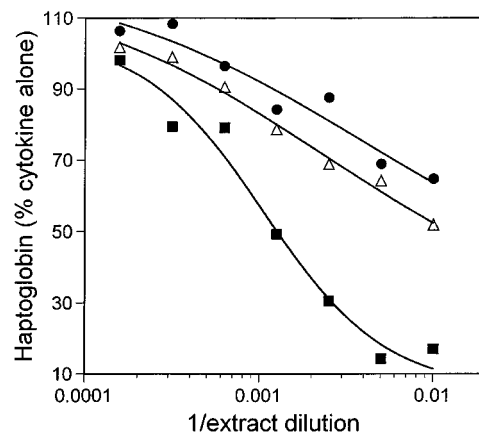


FIG. 4. **HepG2 cell stimulation by other cytokines.** HepG2 cells were incubated with 5 ng/ml IL-6 (■), OSM (△), or LIF (●) in the presence of extract dilutions, and haptoglobin production was expressed as percent of haptoglobin produced in response to each cytokine when no extract was present (37.1 ± 4.4 ng/ml for IL-6, 38.1 ± 2.1 for OSM, and 41.7 ± 5.1 ng/ml for LIF). Each point represents the mean of at least 4 determinations; S.D. was <12% of the mean.

receptor-ligand complexes with gp130 were identical and is blocked by the extract protein, it would be more difficult for an IL-6-IL-6R complex to compete with a gp130 inhibitor and

acquire sufficient interactions with gp130 to complete the signaling circuit than it would be for OSM or LIF. Thus, the extract protein would be expected to be more inhibitory for IL-6 than for OSM or LIF, and this is borne out by our experimental findings.

The overlapping structural relationships of cytokine families and of their corresponding receptors have in the last few years excited much interest and speculation concerning their evolution (2, 7). Sponges are of an ancient lineage of multicellular vertebrates which have existed for about 500 million years. It is quite possible that the *Callyspongia* protein whose activity we describe, which connects with the IL-6 family of cytokines through interaction with gp130, will turn out to be structurally related to receptor or cytokine. Thus, identification of this sponge protein will provide not only a possible lead for a functional inhibitor for these cytokine and receptor families, but may in addition yield valuable information concerning their evolutionary pathways.

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