Receptors for the monokine, interleukin-1 (IL-1), have been successfully immunoprecipitated with a xenogeneic antiserum raised in our laboratories. Receptors solubilized from mouse cell membranes as well as nascent chains of molecules that could bind IL-1 were immunoprecipitated. Receptor complexes were identified on mouse cell lines which express IL-1 receptors by affinity cross-linkage of the radiolabeled ligands, IL-1-α or IL-1-β. Soluble IL-1 or IL-1 nonspecifically associated with membranes of cells which do not express IL-1 receptors was not immunoprecipitated. It is apparent, thus, that antibodies present in the xenogeneic antiserum could specifically bind to the IL-1 receptor moiety within the complex. The major proportion of IL-1 receptor complexes that were reproducibly immunoprecipitated had a molecular weight of 97,000. Cell membrane associated receptors for the monokine, tumor necrosis factor, were not immunoprecipitated. These antibodies have contributed to the understanding of the role of IL-1 receptors in cytolytic effector T cell generation and should contribute further in the purification and characterization of the IL-1 receptor moiety, as well as in determining IL-1-mediated mechanisms of cellular activation.

Interleukin-1 (IL-1) is a polypeptide hormone that is produced by activated macrophages and that plays important roles in the activation of the immune system and inflammatory tissue responses (1–7). The mechanisms by which IL-1 molecules exert their biological effects are not clearly understood; however, it is presumed that IL-1 functions via binding to specific cell surface receptors. Two distinct forms of human IL-1 genes and proteins, IL-1-α (31,000, 17.5 kDa) and IL-1-β (31 70.0, 15–17.5 kDa), have been characterized (8–13). Dower et al. (14) first demonstrated that purified human IL-1-β could be radiolabeled with [35S] and retain its biological activity. The radiolabeled IL-1-β was covalently coupled with affinity homobifunctional cross-linking reagents to its receptor on certain T cell lines and to fibroblast cells (14, 15). Most cells were found to express relatively low numbers of receptors capable of binding IL-1 with high binding affinities. A receptor-ligand complex with a molecular mass of 97 kDa was most consistently observed. The deduced molecular mass for the receptor was 79.5 kDAs. Fibroblast cells expressed an average of 4800 IL-1 receptors/cell and bound IL-1 with an affinity of 2.5 × 10^5 M\(^{-1}\), whereas T lymphoma cells expressed 238 IL-1 receptors/cell and bound IL-1 with an affinity of 3.6 × 10^8 M\(^{-1}\). Murine IL-1-α, human IL-1-α, and human IL-1-β have all been shown to be capable of binding to the same receptor on mouse T-thymoma cells, EL-4 (16). Human Epstein-Barr virus-transformed lymphocytes (EBV-B cells) also bound both human IL-1-α and IL-1-β to the same putative receptor site (17). The molecular mass of the IL-1 receptors isolated from these EBV-B cells, however, was estimated to be 60 kDa. More recently, a mutant subline of EL-4 thymoma cells, EL-4-6.1, was shown to express two classes of IL-1 receptors based on a difference in binding affinities (18). The second class of receptors comprised only 1–2% of the total and bound IL-1 with a 100-fold higher affinity (18). The high affinity IL-1 receptors on these mutant cells were the only receptors able to internalize IL-1 (18). The facts that IL-1 receptors may exist in multiple forms and that different molecular weight assignments have been made for receptors isolated from different cells and in different laboratories suggest that another reagent, that could identify IL-1 receptors, would be most useful for their biochemical characterization. An antiserum that we had previously described (19–22) serves as such a reagent, and in the data presented here we demonstrate that this antiserum immunoprecipitates IL-1 receptor complexes. The fact that we could use radiolabeled ligand to specifically identify IL-1 receptors and that the ligands could covalently bind and tag those receptor molecules from among other cell membrane components that might react with our antisera allowed us to use a polyclonal antiserum to specifically characterize the IL-1 receptor. The antisera immunoprecipitates IL-1 receptors expressed on mouse thymoma, lymphoma, and fibroblast lines (23) but does not immunoprecipitate soluble IL-1 or IL-1 nonspecifically associated with cell surfaces.

In a companion paper (24), we also present evidence that a M\(_r\) 32,000 protein synthesized in vitro from immunoselected mRNA has the capacity to bind to recombinant IL-1 but not to recombinant IL-2 or to TNF-α. The mRNA encoding this IL-1 binding protein was obtained from polysomes that had been purified by immunoadsorbent chromatography utilizing our polyclonal antiserum described below (Miniprint Supplement). The IL-1 binding protein can be immunoprecipitated by our antisera either before it becomes associated with its ligand or upon dissociation from its ligand. These data support the conclusion that the rat anti-SgHF antisera contain antibodies directed against the IL-1 receptor.

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FIG. 1. Immunoprecipitation of IL-1 receptor-ligand (125I-IL-1 complex) with xenogeneic rat anti-SgHF (*) or normal rat immunoglobulin (O). Aliquots of rat anti-SgHF or normal rat serum were incubated with formalin-fixed protein A bearing Staphylococcus aureus conjugated to affinity-purified goat anti-rat immunoglobulin. Aliquots of the resultant conjugates, serum were incubated with formalin-fixed protein A bearing Staphylococcus aureus conjugated to affinity-purified goat anti-rat immunoglobulin. Aliquots of the resultant conjugates, viz. S. aureus rat anti-SgHF immunoglobulin and S. aureus normal rat immunoglobulin, were mixed with lysates made from EL-4 cells cross-linked to 125I-IL-1-α so as to obtain the final dilutions of rat anti-SgHF or normal rat serum in the immunoprecipitation reaction mixture as indicated (0.25 × 10^{-2} = 1:400 dilution; 0.5 × 10^{-2} = 1:200 dilution; 1 × 10^{-2} = 1:100 dilution; 2.5 × 10^{-2} = 1:40 dilution; 5 × 10^{-2} = 1:20 dilution). Each data point represents the mean of four replicate tests. The standard deviation values for immunoprecipitation with rat anti-SgHF ranged from ±112 to ±259, while those for immunoprecipitation with normal rat serum ranged from ±16 to ±24. Counts immunoprecipitated with rat anti-SgHF at concentrations of 5 × 10^{-2}, 2.5 × 10^{-2}, and 1 × 10^{-2} were significantly different from those immunoprecipitated with normal rat serum (p < 0.01 by χ-squared test).

**EXPERIMENTAL PROCEDURES AND RESULTS**

**Immunoprecipitation of IL-1 Receptor-Ligand Complexes**—EL-4 cells were incubated with radiolabeled IL-1-α, then washed, affinity cross-linked, and finally, solubilized in CHAPS buffer. The soluble lysates of these cells which contained ligand-labeled IL-1 receptor (125I-IL-1-α receptor complexes) were then subjected to immunoprecipitation to determine whether the antisera, rat anti-SgHF, recognized the radiolabeled cross-linked receptor-ligand complex. Immunoprecipitation reactions were performed with either rat anti-SgHF conjugate or normal rat immunoglobulin conjugate. The immunoprecipitates from the rat anti-SgHF indeed contained radiolabeled complexes. Immunoprecipitation was dependent upon the concentration of the antisera used in the reaction mixture (Fig. 1). Maximum immunoprecipitation occurred with antisera at dilutions of 2.5 × 10^{-2} (1:40) and 1 × 10^{-2} (1:100). Normal rat immunoglobulins, on the other hand, were unable to immunoprecipitate 125I-IL-1-α-bound IL-1 receptor complexes. These data clearly indicated that the rat anti-SgHF recognized the IL-1 receptor, IL-1-ligand complex.

**Specificity of the Xenogeneic Antiserum for IL-1-labeled Surface Receptors**—Antigenic specificity of the rat anti-SgHF serum for the IL-1 receptor was examined. The P388D1 cell line expresses a relatively high number of receptors for TNF-α. Radiolabeled TNF-α was affinity crosslinked to its receptors on P388D1 cells, and the solubilized cellular components were subjected to immunoprecipitation. IL-1 receptors on EL-4 cells were also affinity cross-linked to 125I-IL-1-α and immunoprecipitated. Electrophoreses of the respective immunoprecipitates and nonprecipitable supernatants in SDS-PAGE revealed that the IL-1-α-radiolabeled IL-1 receptors on EL-4 cells were readily immunoprecipitated with the rat anti-SgHF antiserum (Fig. 3, lane 6). No immunoprecipitate was evident, however, with the TNF-α-radiolabeled receptors on P388D1 cells (Fig. 3, lane 3). In fact, the radiolabeled TNF-α-receptor complex as well as monomeric and dimeric forms of TNF-α (26) remained in the nonimmunoprecipitable supernatant (Fig. 3, lane 2). No radiolabeled IL-1-α-receptor complexes remained in the nonimmunoprecipitable supernatant from IL-1-labeled EL-4 cells (lane 5). The molecular sizes of the TNF-receptor complex, 95 kDa, as well as the multiple forms of TNF observed corresponded with those reported previously by others (26). The major complex immunoprecipitated from IL-1-α-radiolabeled cells was detected at 97 kDa. The autoradiograph also revealed two weaker bands; one was consistent with dissociated IL-1 at about 18 kDa while the second appears to be an additional receptor complex at 116 kDa (Fig. 3, lane 6). The rat anti-SgHF, therefore, contains antibodies that can immunoprecipitate IL-1-α receptors but not TNF receptors.

**Lack of Reactivity against Soluble IL-1 or IL-1 Subjected to Cross-linkage in Solution or on Cells Lacking IL-1 Receptors**—IL-1 receptors radiolabeled with IL-1-β were also subjected to immunoprecipitation reactions. Radiolabeled IL-1-β receptors solubilized from EL-4 cells were immunoprecipitated with the xenogeneic anti-SgHF antiserum (Fig. 6, lane 6).

![Fig. 3. Specificity of the rat anti-SgHF antiserum for IL-1 receptors.](image-url)
16410

Immunoprecipitation of Interleukin-1 Receptors

**Fig. 6. Immunoprecipitation of IL-1 receptors radiolabeled with IL-1-β and affinity cross-linked with dithiobis (succinimidylpropionate).** Lanes 1-3 contain the immunoprecipitated pellets from CHAPS lysates of EL-4 cells. Lanes 1 and 2 were immunoprecipitated with the xenogeneic rat anti-SgHF. Lane 1 contains a sample that was not reduced while the sample in lane 2 was reduced with 2-mercaptoethanol before application to the gel. Lane 3 contains the immunoprecipitate obtained with normal rat serum. Lane 4 contains the rat anti-SgHF immunoprecipitate obtained from WEHI-3 cells incubated with radiolabeled IL-1. Lane 5 contains the rat anti-SgHF immunoprecipitate obtained from EL-4 cells that had also been incubated with unlabeled IL-1-β during the binding reaction with 125I-IL-1-β. Lane 6 contains the immunoprecipitate obtained when rat anti-SgHF was incubated with 125I-IL-1-β which had also been subjected to cross-linkage with dithiobis (succinimidylpropionate).

Two different molecular sized complexes were observed in some experiments. A major band was evident at 97 kDa and a minor band at 116 kDa. The IL-1-β radiolabeled receptor complexes that were immunoprecipitated were identical to those observed upon SDS-PAGE analysis of the solubilized membranes before immunoprecipitation (Fig. 4). Reduction of the immunoprecipitated complexes also resulted in a loss of detectable radiolabeled IL-1 receptor complex, and dissociated IL-1 became readily detectable (Fig. 6, lane 2). No radiolabeled complexes were observed either upon immunoprecipitation with normal rat serum (Fig. 6, lane 3) or from immunoprecipitates of IL-1 receptor negative cells, WEHI-3 (Fig. 6, lane 4). Finally, no immunoprecipitable complex was evident when unlabeled IL-1 was included during the binding reaction (Fig. 6, lane 5) or when cells were excluded from the binding and cross-linking reactions (Fig. 6, lane 6). The xenogeneic antiserum, therefore, did not immunoprecipitate soluble IL-1.

**DISCUSSION**

IL-1 receptors can be immunoprecipitated with xenogeneic rat anti-SgHF, and the data presented here confirm the original hypothesis that antibodies present in this antiserum were directed against a molecule associated with IL-1 function (19, 22). The interactions between IL-1 receptors and the antiserum were demonstrated by the following facts: 1) that immunoprecipitation was dose-dependent with respect to the concentration of rat anti-SgHF; 2) that cold IL-1 added to the binding reaction competitively inhibited both radiolabeled IL-1 binding and the subsequent immunoprecipitation of a radiolabeled receptor complex; 3) that the antiserum did not immunoprecipitate soluble ligand; 4) that the antiserum did not immunoprecipitate either IL-1 nonspecifically associated with cell surface molecules other than its receptors or IL-1 cross-linked to molecules on cells that lacked IL-1 receptors; and 5) that the antiserum clearly distinguished between TNF radiolabeled receptors and IL-1 radiolabeled receptors. It is apparent, thus, that IL-1 receptor-ligand complexes were immunoprecipitated as a result of antibodies present in the xenogeneic rat anti-SgHF that were directed against the IL-1 receptor. These observations also suggest that the IL-1 ligand binding site and the configuration recognized by at least some of these antibodies were distinct determinants on the IL-1 receptor molecules. The antibody combining sites remained available for immunoprecipitation after IL-1 bound to the ligand binding site.

The major species of IL-1 receptor complexes immunoprecipitated from EL-4 cells was 97 kDa. The deduced molecular mass of the major receptor protein itself was approximately 80 kDa. A larger sized minor band was also observed in our studies (Figs. 3-6) and corresponds to a deduced molecular mass for the receptor component of approximately 99 kDa. The fact that we observed two radiolabeled IL-1 receptor complexes is consistent with data reported earlier for the IL-1-β receptor complexes cross-linked under similar conditions (14). It should be noted that the second larger sized but minor band was observed when either IL-1-α or IL-1-β was used to radiolabel the IL-1 receptor (Figs. 3-6). The larger sized IL-1-labeled receptor complex generally appeared as a weaker band with the exception of some experiments in which radiolabeled IL-1 binding occurred in the presence of recombinant IL-2. EL-4 cells do express IL-2 receptors as well as IL-1 receptor molecules. The upregulation of IL-1 receptor expression has been demonstrated to occur following treatment of receptor-bearing cells with glucocorticoids (28). The influence of recombinant IL-2 on IL-1 receptor expression, however, has not been reported. Our data suggest, however, that normally proliferating EL-4 cells, grown in endotoxin-free medium and without activation, express a lesser quantity of one of the IL-1 receptor proteins than of the other. The precise nature of these two IL-1 receptor proteins is not yet known, and we could only speculate at this point that they may either represent different glycosylated forms of a single protein or are distinct proteins which form a single receptor molecule. Scatchard analysis of our binding data indicated the detection of a single class of binding sites with a K_D of 2.5 x 10^-10 M. It would, thus, appear that the two proteins detected are closely related or associated on the cell surface. A total of approximately 1174 IL-1 receptor sites was estimated per EL-4 cell. These figures are in close agreement with those of others (16). Analyses of the immunoprecipitated complexes revealed that the xenogeneic rat anti-SgHF antibodies were able to immunoprecipitate 69% of the IL-1 receptor sites.

The utilization of radiolabeled ligands to tag receptor molecules has led to a substantially greater specificity of cell membrane radiolabeling as compared to direct labeling of cell surface proteins with 125I or metabolic labeling. Although some of the radiolabeled ligand may become nonspecifically associated with cell membranes, the randomness of this nonspecific association does not lead to adequate affinity cross-linkage or the the identification of numerous radiolabeled bands upon SDS-PAGE analysis. The receptor for the ligand, however, becomes specifically labeled; therefore, a concentration of receptor-associated radiolabeled ligands can be identified as a major radioactive band upon SDS-PAGE analysis and autoradiography. Ligand radiolabeling of specific receptors also allows one to use a polyclonal antiserum to identify the specific receptor-ligand complexes since other cell surface
Immunoprecipitation of Interleukin-1 Receptors

Determinants that might be recognized by the antiserum have not become specifically radiolabeled. Furthermore, the numbers of IL-1 receptors expressed per cell represent a very small fraction of surface molecules that would become radiolabeled by direct iodination or metabolic labeling. The specific labeling of the receptor molecules with radioactive ligand, therefore, becomes the most feasible procedure for their identification either in cell lysates or upon immunoprecipitation with a xenogeneic antiserum.

The one problem that arises with the immunoprecipitation of receptors cross-linked to their ligands is the identification of the precise antigenic target of the antisera, the receptor or the ligand. We have clearly demonstrated here that our xenogeneic antiserum does not immunoprecipitate soluble IL-1 nor does it immunoprecipitate IL-1 cross-linked to surface proteins to which it may have become nonspecifically associated during the binding reaction. One might also suggest that the antiserum recognizes only ligand-occupied receptors. Two antiserum inhibited IL-1-mediated functions when the xenogeneic antiserum was incubated with IL-1-responsive cells in the presence of IL-1. Second, in the accompanying paper, we demonstrate that the antiserum can immunoselect nascent peptide chains on polysomes bearing mRNA. The mRNA isolated from these polysomes can be translated into proteins, which have not been post-translationally modified. The translated proteins specifically bind to IL-1 and do not bind to IL-2 or TNF-α. Immunoprecipitation of the translation products resulted in the removal of proteins capable of binding to IL-1. Furthermore, proteins eluted from IL-1 ligand immobilized on a solid phase were still recognized by the antiserum and were successfully immunoprecipitated. Soluble IL-1 receptor molecules, therefore, without bound IL-1, could be immunoprecipitated. We conclude then that the xenogeneic rat anti-SgHF antibodies recognize epitopes on IL-1 receptors. The antigenic determinants on the IL-1 receptors are naturally expressed in the absence of bound ligand as well as in the presence of ligand.

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REFERENCES

1. Oppenheim, J. J., and Potter, M. (1981) in Cellular Functions in Immunity and Inflammation (Oppenheim, J. J., Rosenstreich, D. L., and Potter, M., eds) pp. 1-29, Elsevier/North-Holland Biomedical Press, Amsterdam

2. Oppenheim, J. J., and Gery, I. (1982) Immunol. Today 3, 113-118

3. Dinarello, C. A. (1984) Rev. Infect. Dis. 6, 51-95

4. Smith, K. A., Lachman, L. B., Oppenheim, J. J., and Favata, M. F. (1980) J. Exp. Med. 151, 1551-1556

5. Mizel, S. B., Dayer, J. M., Krane, S. M., and Mergenhagen, S. E. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2474-2477

6. Mizel, S. B. (1982) Immunol. Rev. 63, 51-72

7. Schmidt, J. A., Mizel, S. B., Cohen, D., and Green, I. (1982) J. Immunol. 128, 2177-2182

8. Lomvedico, P. T., Gubler, V., Hellmann, C. P., Dukovich, M., Giri, J. G., Pan, Y. C. E., Collier, K., Seminow, R., Chua, A. O., and Mizel, S. B. (1984) Nature 312, 458-462

9. Schmidt, J. A. (1984) J. Exp. Med. 160, 727-727

10. Auron, P. E., Webb, A. C., Rosenwasser, L. J., Mucci, S. F., Rich, A., Wolff, S. M., and Dinarello, C. A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7907-7911

11. March, C. J., Mosley, B., Larzen, A., Cerretti, D. P., Braedt, G., Price, V., Gillis, S., Henney, C. S., Kronheim, S. R., Gradbatein, K., Conlon, P. J., Hopp, T. P., and Cosman, D. (1985) Nature 315, 641-647

12. Gubler, U., Chua, A. O., Stern, A. S., Hellmann, C. P., Vitak, M. P., DeChiara, T. M., Benjamin, W. R., Collier, K. G., Dukovich, M., Pafmilletti, P. C., Fiedler-Nagy, C., Jenson, J., Kaffka, K., Kilian, P., Stremlo, D., Weissrech, B. H., Woehe, D., Mizel, S. B., and Lomvedico, P. T. (1986) J. Immunol. 136, 2492-2497

13. Kronheim, S. R., March, C. J., Erb, S. K., Conlon, P. J., Mochizuki, D. Y., and Hopp, T. P. (1985) J. Exp. Med. 161, 490-502

14. Dower, S. K., Kronheim, S. R., March, C. J., Conlon, P. J., Hopp, T. P., Gillis, S., and Urdal, D. L. (1985) J. Exp. Med. 162, 501-515

15. Dower, S. K., Call, S. M., Gillis, S., and Urdal, D. L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1069-1064

16. Kilian, P. L., Kaffka, K. L., Stern, A. S., Woehe, D., Mizel, S. B., and Lomvedico, P. T. (1986) J. Immunol. 136, 4509-4514

17. Matsushima, K., Akahoshi, T., Yamada, M., Furutani, Y., and Oppenheim, J. J. (1986) J. Immunol. 136, 4496-4502

18. Lowenthal, J. W., and MacDonald, H. R. (1986) J. Exp. Med. 164, 1090-1074

19. McMannis, J. D., and Plate, J. M. D. (1985) Transplantation 40, 405-412

20. Plate, J. M. D., McDaniel, C. A., Fischert, L., Stimphling, J. H., Melvold, R. W., and Martin, N. Q. (1982) J. Exp. Med. 155, 681-697

21. Plate, J. M. D. (1984) Proc. Am. Assoc. Cancer Res. 25, 264

22. McMannis, J. D., and Plate, J. M. D. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1513-1517

23. Plate, J. M. D., and Rangnekar, V. M. (1988) Prog. Leukocyte Biol. 8, 167-174

24. Rangnekar, V. M., and Plate, J. M. D. (1988) J. Biol. Chem. 263, 16414-16420

25. Aggarwal, B. B., Essalou, T. E., and Hass, P. E. (1986) Nature 318, 665-667

26. Kull, F. C., Jr., Jacob, S., and Custreuccas, P. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5756-5760

27. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672

28. Matsushima, K., Kobayashi, Y., Copeland, T. D., Akahoshi, T., and Oppenheim, J. J. (1987) J. Immunol. 139, 3367-3374

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RESULTS

Efficiency of Immunoprecipitation of Labeled Radiolabeled IL-1 Receptors

Immunoprecipitation was performed to determine the efficiency of the immunoprecipitation of labeled radiolabeled IL-1 receptors. The results were analyzed by densitometry of the autoradiograms. The data showed that the efficiency of immunoprecipitation was significantly higher than the control group. The results were statistically analyzed using the Student's t-test and the difference was significant (p < 0.05).



In summary, the results obtained in this study indicate that the immunoprecipitation of labeled radiolabeled IL-1 receptors is efficient and can be used as a tool for studying the binding of IL-1 receptors to their ligands. The results also suggest that the immunoprecipitation method is a useful approach for studying the function of IL-1 receptors in various biological systems.
**Immunoprecipitation of Interleukin-1 Receptors**

**FIGURE 1.** Affinity cross-linking of IL-1-beta with DST either in the presence or in the absence of cells. Lanes 1 and 6 contain CHAPS lysates of EL-4 cells that had been incubated with 125I-IL-1-beta and cross-linked before solubilization. Lane 1 was not reduced while lane 2 was reduced with 2-mercaptoethanol before SDS-PAGE analysis. Lanes 2 contains CHAPS lysates of EL-4 cells that had also been incubated with unlabeled IL-1 during the binding reaction of 125I-IL-1-beta. Lane 3 contains CHAPS lysates of MD-3 cells that had been incubated with 125I-IL-1-beta and subjected to cross-linking before solubilization. Lanes 4 and 5 contain soluble IL-1-beta and IL-2, respectively, which had been subjected to cross-linking in solution. The gels were exposed to Kodak X-Omat film at -70°C for three weeks.

**FIGURE 2.** Specificity of 125I-beta binding to surface receptors. Lane 1 contains EL-4 cells incubated with 125I-IL-1-beta in the presence of 125I-beta before cross-linkage with DST. Lane 1 contains EL-4 cells incubated with 125I-IL-1-beta and cross-linked with the irreducible cross-linking reagent, DST. The CHAPS lysates in Lane 1 were reduced with 2-mercaptoethanol before application to the gel. The gels were exposed to Kodak X-Omat film at -70°C. Lane 1 was on a gel exposed for three days, while lane 2 was from a gel exposed for three weeks.