The interleukin-1 receptor in Raji human B-lymphoma cells

Molecular characterization and evidence for receptor-mediated activation of gene expression

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In a previous paper [Horuk, Huang, Covington & Newton (1987) *J. Biol. Chem.* **262**, 16275–16278] we reported that there were fundamental differences in the biochemical properties of the interleukin-1 (IL-1) receptor between Raji and EL4 cell lines. In the present study we have investigated the basis for these differences. Kinetic studies measuring the on and off rates of IL-1 receptor binding revealed that the low-affinity IL-1-binding sites observed in Raji cells, compared with EL4 cells, result from a combination of a lower association rate and a higher dissociation rate in the Raji cells. The turnover of the Raji IL-1 receptor, measured by inhibiting protein synthesis with cycloheximide, was much faster than that of the EL4 IL-1 receptor, with a half-time of 2 h as against 5 h. Treatment of ^{125}I -IL-1-labelled IL-1 receptors in Raji and EL4 cells with neuraminidase decreased their molecular mass by approx. 2–5 kDa as assessed by SDS/polyacrylamide-gel electrophoresis (PAGE). The covalently labelled IL-1 receptors in both cell types were sensitive to treatment with endoglycosidase F, which decreased their molecular mass on SDS/PAGE by 12–13 kDa. Incubation of Raji cells with maximally stimulating doses of IL-1 resulted in an increase in the nascent RNA levels of several genes, including the IL-2 receptor and the proto-oncogenes *c-Ha-ras* and *c-myc*.

INTRODUCTION

IL-1 is a cytokine, primarily produced by monocytes, that displays a wide spectrum of biological activities in a diverse number of cell types [1]. As well as exerting immunoregulatory activities [2–4], IL-1 is well established as a mediator of inflammation and is involved in the development of rheumatoid arthritis [5]. Numerous reports (cited in [6]) have demonstrated that IL-1 elicits its biological effects by binding to specific cell-surface receptors. A variety of evidence from direct binding and competition studies with radiolabelled IL-1 alpha and beta has suggested that the IL-1-receptor binding site is remarkably well conserved [7–9]. In addition, chemical labelling of IL-1 receptors with radiolabelled IL-1 resulted (after subtraction of the molecular mass of IL-1) in the specific labelling of proteins of molecular mass 80–85 kDa in a variety of cell types examined, suggesting conservation of the structure of the protein [8,10,11]. However, IL-1 receptors having a molecular mass of 60–68 kDa have also been identified by covalent labelling with IL-1 [12,13].

Recently, using an approach consisting of kinetic analysis, competitive radioligand binding studies and covalent labelling of the IL-1 receptor, we provided evidence for fundamental differences in the biochemical properties of the IL-1 receptor in Raji and EL4 cells [14]. We reported that the K_D for IL-1 binding to Raji cells was at least one order of magnitude lower than that to EL4 cells, the internalization rate of the Raji IL-1 receptor was lower and its molecular mass, 14 kDa, smaller than that of the EL4 IL-1 receptor. Several questions arose from these studies: (i) what is the basis for the observed

differences in the affinity of IL-1 binding to its receptor? (ii) do the observed differences in molecular mass of the IL-1 receptors reflect differences in post-translational processing? (iii) is the IL-1 receptor in Raji cells biologically active despite its altered molecular properties? In the present study we have addressed these questions by further characterization of the IL-1 receptor. We show that: (a) the low-affinity IL-1 binding sites observed in Raji cells, compared with EL4 cells, result from a combination of a lower association rate and a higher dissociation rate in the Raji cells; (b) the differences in molecular masses of the IL-1 receptors most probably derive from differences in the polypeptide domains of the receptor; and (c) IL-1 stimulates gene expression presumably via the IL-1 receptor.

EXPERIMENTAL PROCEDURES

Materials

Human recombinant-derived IL-1 beta expressed in *Escherichia coli* was purified to homogeneity according to methods previously described [15]. ^{125}I -IL-1 (sp. radioactivity 143–180 $\mu\text{Ci}/\mu\text{g}$), labelled with the Bolton–Hunter reagent, was from New England Nuclear. Reagents for electrophoresis were from Bio-Rad. Hepes and all other reagent-grade chemicals were from Sigma.

Methods

Cell culture. Raji and EL4 cells were obtained from the American Type Culture Collection (A.T.C.C.) and were maintained in RPMI 1640 medium containing 10% (v/v) fetal-calf serum. The cells were passaged weekly,

and the medium was changed two additional times weekly. For binding assays the cells were collected, washed three times with RPMI 1640 and resuspended in binding buffer (RPMI 1640 containing 1 % bovine serum albumin and 20 mM-Hepes, pH 7.2). Some incubations were carried out in the presence of 0.2 % sodium azide. Cell viability was assessed by Trypan Blue exclusion, and cell number was determined by counting the cells in a haemocytometer.

Receptor binding assay of ^{125}I -labelled IL-1 to intact cells. Raji and EL4 cells (2×10^7 cells/ml) were incubated with various concentrations (50 pM–1 nM) of ^{125}I -labelled IL-1 at 37 °C for the designated times. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicone/paraffin oil mixture as described previously [16]. Non-specific binding was determined in the presence of 1 μM unlabelled IL-1. Unless otherwise indicated, all incubations were carried out for 3 h in the presence of sodium azide (0.2 %) to inhibit internalization and degradation of ^{125}I -labelled IL-1. The biological activity of the radiolabelled IL-1 was the same as that of unlabelled IL-1 (results not shown).

Association and dissociation studies. For association kinetics experiments, cells in RPMI buffer were incubated with various concentrations of radiolabelled IL-1 as indicated above. At predetermined times, aliquots were removed, centrifuged through oil and the cell pellets counted for radioactivity as described above. For dissociation kinetics, cells were incubated with 500 pM radiolabelled IL-1 in the presence or absence of 1 μM unlabelled IL-1 for 3 h at 37 °C. Cells were then centrifuged and resuspended in the same volume of RPMI buffer containing 1 μM unlabelled IL-1. The time at which the cells were resuspended was taken as zero time. The cells were then incubated at 37 °C, and aliquots were taken at various times for counting for radioactivity as described for the association experiments.

Chemical cross-linking of cells and SDS/polyacrylamide-gel electrophoresis (PAGE). Cells (2×10^7 cells/ml) were covalently cross-linked to ^{125}I -labelled IL-1 as previously described [14]. After cross-linking the cells were solubilized and analysed by SDS/PAGE [14].

Neuraminidase and endoglycosidase F treatment of cells. Cells covalently cross-linked to ^{125}I -labelled IL-1 were incubated with 1 unit of neuraminidase (Sigma) in 1 ml of 100 mM-acetate buffer, pH 6.0, for 2 h at 37 °C. For endoglycosidase F treatment, covalently IL-1-cross-linked cells were incubated with 1 unit of the enzyme (Boehringer) in 0.1 ml of 50 mM-phosphate buffer, pH 6.1, containing 0.1 % SDS, 0.1 % Triton X-100, and 1 % mercaptoethanol for 6–8 h at 37 °C. The proteinase inhibitors pepstatin, leupeptin and aprotinin (5 $\mu\text{g}/\text{ml}$) were present throughout the course of the experiments. At the end of the incubation, samples were subjected to SDS/PAGE as described above.

Nuclear transcription. Cells (5×10^7) were incubated with 1 nM-IL-1 beta for 24 h at 37 °C. Nuclei were isolated by density-gradient centrifugation as previously described [17]. The isolation of nascent RNA and nuclear transcription were performed as described by Groudine

et al. [18]. Nuclear-transcription hybridization was carried out as described in [19]. The human IL-2-receptor probe was obtained from Dr. Richard Robb (du Pont) [20]; the human proto-oncogenes *c-Ha-ras* [pbc-N1] [21] and *c-myc* [pHSR-1] [22] were obtained from the A.T.C.C. The human *c-fms* plasmid [RPN-1326X] was purchased from Amersham International. Nuclear-transcription experiments were carried out at least three times, and the intensity of hybridization was determined by densitometric scanning with an LKB Ultrascan laser densitometer. The fold induction of the nascent RNAs by IL-1 was calculated relative to control levels. Under the same conditions IL-1 did not induce the expression of β -actin.

Data analysis. Equilibrium binding data were analysed by using the non-linear regression program LIGAND [23] as adapted for the IBM personal computer by McPherson [24] from Elsevier-BIOSOFT.

RESULTS

The binding of ^{125}I -IL-1 to EL4 and Raji cells at 37 °C as a function of time is shown in Fig. 1. The specific binding of radiolabelled IL-1 reached steady state between 180 and 240 min in both cases. Separate experiments demonstrated that specific binding decreased to ~80 % of maximum after 6 h of incubation (results not shown). This was presumably due to receptor or radioligand degradation. The half-time of association of ^{125}I -IL-1 binding to EL4 and Raji cells was 49 and 77 min respectively (Fig. 1). Semilogarithmic transformations of initial association data yielded linear plots in both cell lines, consistent with association to a homogeneous class of specific binding sites (Fig. 1, insets). The experimentally observed association rate constants (K_{obs}) for IL-1-receptor binding were determined from the slope of the line of the insets to Fig. 1 and were 0.014 (Raji) and 0.009 min^{-1} (EL4) respectively. The association rate constant, K_1 , was derived from k_{obs} , as described in Table 1.

The dissociation of ^{125}I -IL-1 from IL-1 receptors in EL4 and Raji cells in the presence of 1 μM -unlabelled IL-1 is shown in Fig. 2. Dissociation of radiolabelled IL-1 from EL4 cells was remarkably slow, with 50 % of initial binding remaining after almost 6.5 h at 37 °C. The rate constant for dissociation calculated from the data in Fig. 2(a) was $1.6 \times 10^{-3} \text{ min}^{-1}$ (Table 1). The dissociation of ^{125}I -IL-1 was more rapid in Raji cells, half-maximal dissociation occurring after only 96 min (Fig. 2b); however, the deviation from linearity of the dissociation data suggested some heterogeneity in the specific binding sites. Indeed our results indicated that a statistically better fit of the data was obtained by assuming a two-component exponential curve [24]. The initial rapidly dissociating component in Raji cells comprises approx. 78 % of the total complex and has a dissociation rate of $1.1 \times 10^{-2} \text{ min}^{-1}$ (Table 1). The second, slower-dissociating, component (22 %) has a dissociation rate of $3.5 \times 10^{-6} \text{ min}^{-1}$. As shown in Table 1, K_D values calculated as the ratio of kinetic constants K_2/K_1 (for Raji cells K_2 is the dissociation rate constant of the major rapidly dissociating pool and K_1 is estimated as described in Table 1) are 1.5 nM and 106 pM for Raji and EL4 respectively, values that are in reasonable agreement with the K_D determined from steady-state binding

Fig. 1. Association kinetics of ^{125}I -IL-1 binding to (a) EL4 and (b) Raji cells

Cells and radiolabelled IL-1 were incubated in the presence or absence of excess unlabelled IL-1 at 37 °C for the times indicated. At that time aliquots were removed, centrifuged through oil and the cell pellets counted for radioactivity. The insets show semilogarithmic plots of association data. The data are plotted as $\ln[B_0/(B_0 - B)]$ versus association time, where B_0 is specific binding at steady state and B is specific binding for the association time shown.

Table 1. Kinetic parameters of ^{125}I -IL-1 binding to Raji and EL4 cells

Association and dissociation experiments were carried out as described for Figs. 1 and 2. The dissociation constant, K_2 , was determined from the slopes of semi-logarithmic plots of dissociation data. The association rate constant, K_1 , was determined from the equation:

$$K_1 = (K_{\text{obs.}} - K_2)/[L]$$

where $K_{\text{obs.}}$ is the slope of the semi-logarithmic plot of initial association data and $[L]$ is the radioligand concentration. Results are means for two independent determinations (individual values from the two experiments are given in parentheses).

Cells	K_1 ($\mu\text{M}^{-1} \cdot \text{min}^{-1}$)	K_2 (min^{-1})	Kinetically derived K_D (K_2/K_1) (pM)	Steady-state- derived K_D (pM) (from [14])
Raji	7.2 (6.4, 8.0)	1.1×10^{-2} * (1.3×10^{-2} , 0.9×10^{-2})	1520	2100
EL4	15.0 (13.5, 16.5)	1.6×10^{-3} (1.4×10^{-3} , 1.8×10^{-3})	106	400

* For Raji cells dissociation was best described by a two-component system; for convenience only the rate constant of the faster-dissociating component, which comprises 78 % of the total complex, is given in the Table (see the text for an explanation).

(Table 1). Dissociation of radiolabelled IL-1 from EL4 and Raji cells in the presence of 100-fold excess buffer, in the absence of unlabelled IL-1, followed a time course (results not shown) similar to that shown in Fig. 2, suggesting that the rate of dissociation of IL-1 from its receptor is insensitive to receptor occupancy.

The turnover of the IL-1 receptor in EL4 and Raji cells was measured by incubating the cells in the presence of cycloheximide (10 $\mu\text{g}/\text{ml}$) to inhibit protein synthesis. Aliquots were removed and assayed for ^{125}I -IL-1 binding after various time intervals (Fig. 3). The turnover rate of the IL-1 receptor in EL4 cells was fairly low and took almost 5 h for half of the receptors to be degraded, which is in close agreement with the findings of previous studies [25]. In contrast, the turnover time of the IL-1 receptor in Raji cells was higher, with a half-time of degradation of 2.5 h. In addition, the curvilinearity of the data

suggested a more complex process of IL-1 receptor processing than in the EL4 cells. Raji cells incubated in the presence of azide showed kinetics identical with those shown by cells incubated in the absence of azide (results not shown).

To assess whether IL-1 receptors in these two cell types were differentially sialylated, we incubated cells that had been covalently labelled with ^{125}I -IL-1 with neuraminidase as described in the Experimental procedures section. At the end of the incubation the cells were washed, solubilized and subjected to SDS/PAGE. An autoradiogram from a typical experiment is shown in Fig. 4. As shown in Fig. 4, IL-1 is covalently linked to a protein of molecular mass 80 kDa in EL4 cells and 68 kDa in Raji cells, in agreement with results from previous studies [14]. Treatment with neuraminidase decreases the molecular mass of these labelled proteins

Fig. 2. Dissociation of ^{125}I -IL-1 binding to (a) EL4 and (b) Raji cells

Cells and radiolabelled IL-1 were incubated in the presence or absence of excess unlabelled IL-1 at 37 °C for 3 h to attain steady-state binding. At that time the cells were centrifuged and rapidly suspended in the same volume of buffer containing 1 μM -IL-1. Samples were then incubated for the indicated times before centrifugation through oil and counting for radioactivity. The inset shows a semi-logarithmic plot of the dissociation data. The data are plotted as $\ln(B/B_0)$ versus dissociation time, where B_0 is specific binding at steady state and B is specific binding for the dissociation time shown.

by ~2–5 kDa. These data demonstrate that the IL-1 receptor in both cell types is terminally glycosylated with sialic acid. Thus it was of interest to determine whether there were any differences in the degree of glycosylation of the IL-1 receptor that would account for the observed differences in molecular mass. Accordingly we incubated cells that had been covalently labelled with ^{125}I -IL-1 with endoglycosidase F as described in the Experimental procedures section. As shown in Fig. 5, treatment of radiolabelled IL-1 receptor with endoglycosidase F decreased its molecular mass by ~12–13 kDa in both cell types.

The stimulatory effects of IL-1 on gene expression are well documented [26,27] and, in view of the differences in biochemical properties of the Raji IL-1 receptor, it was of interest to examine the effect of IL-1 on gene expression in these cells. Briefly, we incubated cells with saturating

Fig. 3. Turnover of IL-1 receptors in (●) EL4 and (○) Raji cells

Cells were incubated in the presence of cycloheximide (10 $\mu\text{g}/\text{ml}$) for various periods at 37 °C. Aliquots of the cells were then removed and incubated for 3 h at 37 °C with radiolabelled IL-1 in the presence or absence of unlabelled IL-1. The viability of the cells incubated in the presence of cycloheximide did not differ from the control cells incubated in the absence of the drug. Specific binding to cycloheximide-treated cells is expressed as a percentage of the binding to untreated cells assayed in parallel. Binding experiments with EL4 cells were carried out in the presence of 0.2% sodium azide as described in the text.

Fig. 4. SDS/PAGE analysis of IL-1 receptors in EL4 and Raji cells covalently cross-linked with ^{125}I -IL-1 treated with and without neuraminidase

Cells were covalently labelled with radiolabelled IL-1 and incubated in the presence or absence of neuraminidase (1 unit) for 2 h at 37 °C. Control cells were incubated in the absence of enzyme for the stated time. The molecular masses (M) indicated in the left margin were estimated from radiolabelled standards. Abbreviations: IL-1, excess unlabelled IL-1; Neur., neuraminidase.

Fig. 5. SDS/PAGE analysis of IL-1 receptors in EL4 and Raji cells covalently cross-linked with ^{125}I -IL-1 and treated, or not treated, with endoglycosidase F

Cells were covalently labelled with radiolabelled IL-1 and incubated in the presence or absence of endoglycosidase F (1 unit) for 6 h at 37 °C. Control cells were incubated in the absence of enzyme for the stated time. The molecular masses (M) indicated in the left margin were estimated from radiolabelled standards. Abbreviations: IL-1, excess unlabelled IL-1; Endo F, endoglycosidase F.

doses of IL-1 for 24 h at 37 °C and measured the nascent RNA levels of a variety of oncogenes and cellular proteins. We found that IL-1 induced an increase in the nascent RNA levels of several proteins, including the IL-2 receptor and the proto-oncogenes *c-myc* and *c-Ha-ras* (Fig. 6).

DISCUSSION

The kinetic data obtained from ^{125}I -IL-1 binding to Raji and EL4 cells are summarized in Table 1. The value for the association rate constant, K_1 , in EL4 cells reported here is in reasonable agreement with that from other studies [6,7,28]. The kinetic data demonstrate that the basis for the IL-1 binding sites in EL4 being of higher affinity than those of Raji cells is due to a combination of a higher 'on' rate and a lower 'off' rate for IL-1 binding in the EL4 cells (Table 1). The low dissociation rate for IL-1 binding in EL4 cells is consistent with that previously reported [6,7,28]; indeed, at 4 °C, binding of IL-1 to receptors in EL4 cells has been shown to be almost irreversible [7]; no satisfactory explanation for this observation has so far been found. Interestingly, the dissociation of ligand from the D2 dopamine (3,4-dihydroxyphenethylamine) receptor has also been found to be extremely slow, and it has been suggested that this could represent additional binding interactions of the dissociating ligand to a receptor domain distinct from that initially involved in binding [30]. It is tempting to speculate that the presence of accessory binding sites as described above for the dopamine receptor could also

Fig. 6. IL-1-induced stimulation of nascent RNA for various proteins in Raji cells

Raji cells were incubated in the presence or absence of IL-1 (1 nM) for 24 h at 37 °C. Cell nuclei were isolated, and nascent RNA for a variety of proteins was measured by nuclear transcription and hybridization with the appropriate nucleic acid probes. Data are expressed relative to nascent RNA observed in cells incubated in the absence of IL-1. The vertical bar represents the S.E.M. (the data are from three separate experiments).

account for the extremely slow dissociation of IL-1 in EL4 cells.

The turnover of the Raji IL-1 receptor, measured by inhibiting protein synthesis with cycloheximide, was much faster than that of the EL4 IL-1 receptor, with a half-time of 2 h as against 5 h (Fig. 3). Furthermore, the rapid down-regulation of IL-1 receptors by IL-1 observed in EL4 cells [25,28] contrasts strongly with our findings that Raji cells treated with IL-1 do not appear to down-regulate their receptors even after 24 h of incubation (R. Horuk, unpublished data). Taken together, these findings suggest that the intracellular trafficking of the IL-1 receptor in these two cell lines is different and probably subject to disparate regulatory processes.

Elucidation of the structural and molecular properties of IL-1 receptors is an important step toward understanding the biochemical mechanisms involved in IL-1 action. Consequently we have employed chemical cross-linking studies to label the IL-1 receptor in Raji and EL4 cells. The susceptibility of the cross-linked IL-1 receptor in both cell types to treatment with neuraminidase and endoglycosidase F (Figs. 4 and 5) suggests that the IL-1 receptor is a glycoprotein terminally capped with sialic acid. In line with our findings, Bron & MacDonald [31] recently reported that the IL-1 receptor in EL4 cells was an 82 kDa *N*-linked glycoprotein that was converted into a 60 kDa polypeptide after treatment with endoglycosidase F. In addition, Urdal *et al.* [29] have also reported that the 82 kDa affinity-purified IL-1 receptor from EL4 cells could be digested to yield protein bands of 62 and 53 kDa respectively. Our finding that endoglycosidase F treatment of radiolabelled IL-1 receptors in Raji and EL4 cells decreased their molecular masses to the same extent makes it unlikely that the different molecular masses of the IL-1 receptors in these two cell types are due to differences in glycosylation. It is of course possible that differences in glycosylation do exist and that these are masked by the different sensitivities of

the two proteins with the endoglycosidase F. In addition, we cannot rule out the possibility that other post-translational modifications account for the differences in the molecular masses of the IL-1 receptors. It is unlikely that these differences in molecular mass reflect partial proteolysis of the Raji receptor, because the molecular mass of the IL-1 receptor in EL4 cells was not altered by incubation with Raji-cell supernatants (results not shown), indicating that there was no proteinase activity in the extracellular medium capable of converting the IL-1 receptor into a lower-molecular-mass form. It is tempting to speculate that the differences in molecular mass of the IL-1 receptors reflect differences in the polypeptide domains of the two proteins. However, direct confirmation of this hypothesis will require biosynthetic labelling experiments and the availability of receptor-specific antisera.

An obvious question relating to the altered properties of the Raji IL-1 receptor was to determine whether it was capable of mediating any biological effects. In order to test this, Raji cells were treated with stimulating doses of IL-1, nascent RNA was extracted, elongated *in vitro* in the presence of [³²P]UTP, and hybridization studies with a variety of gene probes was carried out. These experiments revealed that IL-1 treatment of Raji cells stimulated gene expression of a variety of proteins, including the IL-2 receptor and the proto-oncogenes *c-Ha-ras* and *c-myc* (Fig. 6). Thus, by the induction of these specific messages, it appears that the IL-1 receptor is biologically active and capable of mediating some biological effects. This does not, however, imply that the altered properties of the IL-1 receptor are inconsequential with respect to its biological properties. Indeed, one of us has been consistently unable to induce surface Ig expression in these cells, even with excess IL-1 concentrations (R. Horuk, unpublished work). These data imply that Raji cells, which are undifferentiated B-cells [32], have lost the ability to switch on the pathways of differentiation that culminate in the expression of surface Ig. The induction of Ig synthesis in B-cells is a complex process under the co-ordinate control of cytokine cascades [33]. Thus it is tempting to speculate that the failure of IL-1 to stimulate Ig synthesis in Raji cells might be partly due to defective coupling of the IL-1 receptor to the transducing unit responsible for mediating these effects of the hormone. This could in turn be related to the altered molecular properties of the IL-1 receptor described above.

The stimulatory effects of IL-1 on gene expression of *c-Ha-ras* and *c-myc* are interesting. *ras*-oncogene products share some structural and functional properties with GTP-binding proteins [34] and have been implicated in the regulation of cell growth and differentiation [35]. Therefore it is possible that *ras* proteins may act as a relay between cell-surface receptor binding and the activation of intracellular events. Although it remains to be established whether IL-1 action is mediated via an adenylate cyclase-linked signal-transduction pathway, recent work suggests that IL-1 appears to mediate synovial-cell activation via increases in cyclic AMP [36]. In contrast with the *ras* proteins, peptides encoded by the *c-myc* oncogene are localized in the nucleus and appear to be transcriptionally activated after hormonal stimulation [37]. Enhanced *c-myc* expression has also been found in normal B-lymphocytes after their activation with lipopolysaccharide [38], and a role for *c-myc* in the regulation of the cell cycle has been proposed [38].

These studies demonstrate that IL-1 receptors in Raji and in EL4 cells are different in their biochemical properties and support the notion that at least two different types of IL-1 receptor exist. In line with this idea a recent report [39] suggests that rat hypothalamus has IL-1 receptors that recognize IL-1 beta but not IL-1 alpha. In addition, Rosoff *et al.* [40] have recently suggested that IL-1 is able to stimulate diacylglycerol production in two T-cell lines, EL4 and Jurkat; however, no IL-1 binding was detected in the Jurkat cells. Clearly then, cells may have evolved not only different IL-1 receptors but also different mechanisms for the propagation of IL-1 action.

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