Internalization of Human Interleukin 4 and Transient Down-regulation of Its Receptor in the CD23-inducible Jijoye Cells*

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Human interleukin 4 (IL-4) specifically induces the low affinity receptor for IgE (FcεR2/CD23) on the surface of the Burkitt lymphoma cell line Jijoye. At 4 °C 125I-IL-4 specifically binds to high affinity receptors (K_d = 4–10 × 10^{-11} M; B_max 600–1,200 sites/cell). Following a rapid temperature shift from 4 to 37 °C, 80% of the receptor-bound 125I-IL-4 disappeared from the cell surface within 20 min (t_1 = 8.9 min). For every two internalized molecules of IL-4 (t_1 = 13 min), one molecule of IL-4 dissociated from the cell surface (t_0 = 25 min). More than 90% of the internalized IL-4 was released in a degraded form into the medium following 25 min. More than 90% of the internalized IL-4 was released in a degraded form into the medium following 25 min. The reexpression of the IL-4R may be an important step necessary for IL-4 induction of the FcεR2/CD23.

EXPERIMENTAL PROCEDURES

RESULTS

Internalization and Degradation of 125I-IL-4 at 37 °C—We previously demonstrated that 125I-IL-4 specifically binds with a high affinity (K_d = 40–100 pM) to Jijoye cells at 4 °C (12). Each cell expressed an average of 600–1,200 binding sites. 125I-IL-4 bound to cells at 4 °C was localized on the cell surface since 90–95% of the radioactivity could be eluted by acid treatment (not shown). Here, experiments were designed to study the fate of cell surface receptor-bound 125I-IL-4 following a temperature shift from 4 to 37 °C (Fig. 1). Within 20 min of incubation at 37 °C, approximately 80% of the cell surface receptor-bound 125I-IL-4 disappeared (t_0 = 8.6 min). By 90 min no cell surface 125I-IL-4 was detectable, and concomitantly, there was an increase in the concentration of fragmented IL-4 which reached a maximum at 30 min (60–65% of the initial cell surface receptor-bound 125I-IL-4) but then declined to near background levels by 180 min. Intact dissociated 125I-IL-4 also appeared in cell supernatant reaching a maximum of 35–40% at 60 min. A significant amount of degraded 125I-IL-4 was observed in supernatants by 30 min (7% of the initially bound IL-4), and this level increased steadily, as concomitantly the level of internalized 125I-IL-4 declined. Fig. 2 shows that sodium azide, cytochalasin B, and lysosomotropic agents (NH_4Cl, methylamine, monensin, and chloroquine) were able to inhibit 125I-IL-4 degradation by 80–100%. Since Jijoye cell-conditioned medium is not able to degrade 125I-IL-4 over a 24-h incubation time (Fig. 2, supernatant), the results demonstrate that the degradation of 125I-IL-4 is a consequence of the ligand internalization. The data for the dissociation (P) and internalization (I) of 125I-IL-4 can be treated as a system
of dissociated intact IZ5I-IL-4 versus 1 appeared from the surface with first order kinetics and a rate constant 37 ml) were incubated for 3 h at 4°C with an excess of unlabeled reactant (P = 1000 cpm/ml). After a rapid temperature shift from 4 to 37°C (t = 0), the respective amounts of cell surface receptor-bound (A), internalized (I), dissociated (D), and released degraded (Δ) IZ5I-IL-4 were measured as described under "Experimental Procedures." All these values are expressed as percentages of the cell surface receptor-bound IZ5I-IL-4 at time 0 and are the means ± S.E. of three independent experiments, each point in triplicate.

Fig. 3A shows that 80% of receptor-bound IZ5I-IL-4 disappeared from the surface with first order kinetics and a rate constant, k = 1.3 × 10^-5 s^-1 (t_0 = 8.9 min). Moreover, a plot of dissociated intact IZ5I-IL-4 versus 1 - e^{-k(t-t_0)} (Fig. 3B) is linear with an intercept close to 0. This indicates that the dissociation of intact IZ5I-IL-4 is also a first order process with an estimated k_{off} = 4.7 × 10^-4 s^-1 (t_0 = 55 min). The rate constant, k_{in} (rate of IZ5I-IL-4 internalization), was calculated to be 8.8 × 10^-4 s^-1 (t_0 = 13 min). k_{in} versus k_{off} can also be established by calculating the ratio I/P = 1.7-1.6 which is close to the ratio k_{in}/k_{off} = 1.9. These results indicate that two competitive first order processes exist: (a) internalization and (b) dissociation of the lymphokine from receptor. Finally, release of degraded IZ5I-IL-4 was also found to follow first order kinetics with a t_0 = 68 min (k = 1.7 × 10^-4 s^-1) (Fig. 3C).

Modulation of the Cell Surface IL-4 Receptor Expression by IL-4—Having demonstrated that IL-4 was internalized by Jijoye cells, we wondered whether a subsequent modulation of the expression of the cell surface IL-4R occurred. To this end, Jijoye cells were cultured at 37°C with an excess of unlabeled IL-4. Residual IL-4R were measured by binding of IZ5I-IL-4 after acid elution of cell surface-bound unlabeled IL-4. Addition of unlabeled IL-4 (1 nM) results in a rapid decrease of the IL-4R levels (Fig. 4A). A nadir is reached after 2 h when 75% of the cell surface receptors have disappeared. This decrease of IL-4R did not occur when cells were incubated at 4°C with 1 nM unlabeled IL-4, demonstrating that down-regulation of the IL-4R is temperature-dependent. Scatchard analysis of equilibrium saturation binding data at times 0 and 2 h revealed that the decrease of cell surface-bound IZ5I-IL-4 was due to a decrease of the number of binding sites/cell from 960 (time 0) to 330 (time 2 h) and not to a change in the affinity constant, K_0 = 45 pm (time 0), K_0 = 59 pm (time 2 h) (Fig 4B).

Interestingly, following a 2-h incubation designed to down-regulate the receptor, we found that the IL-4R was reexpressed, in spite of the presence of an excess of IL-4. Jijoye cells cultured for 0 or 24 h with IL-4 or 24 h without IL-4 displayed IZ5I-IL-4 with a similar K_0 of 45, 31 (Fig. 4B), and 50 pm (not shown), respectively, and also a similar number of binding sites/cell of 960, 820 (Fig. 4B), and 900 (not shown). The expression of IL-4R after 24 h of culture was not due to a decreased ability of the remaining IL-4 to induce receptor down-regulation or internalization: (i) after 24 h of incubation with the cells, the IL-4-containing supernatant was still able to down-regulate (75% at 2 h) IL-4R on another cell batch (not shown); (ii) since the intracellular pool of IZ5I-IL-4 remains constant between 13 and 24 h, the rate of IZ5I-IL-4 internalization between 13 and 24 h is equal to the rate of degradation and represents 0.73 molecules/s (2.5% of IZ5I-IL-4 is degraded; Fig. 5). This value is comparable to the rate of internalization calculated when cells encountered IL-4 for the first time: V_{in} = k_{in} × S = 8.8 × 10^-1 × 969 = 0.84 molecules/s.

Reexpression of IL-4R Requires DNA Transcription and Protein Synthesis—The reexpression of cell surface IL-4R was further investigated by culturing Jijoye cells with 1 nM IL-4 together with agents able to block either protein synthesis, DNA transcription, or receptor recycling. The reappearance of IL-4R was inhibited by cycloheximide (CHX) which blocks protein synthesis and actinomycin D which blocks DNA transcription (Fig. 6). CHX was a strong inhibitor since cells cultured for 1-2 h with IL-4 and CHX expressed less IL-4R than cells cultured with IL-4 alone, and under these conditions reappearance of IL-4R after 2 h could not be seen. These data indicate that protein synthesis is required very early in the reexpression of IL-4R. Chloroquine which is known to block receptor recycling (25) delays the reappearance of the IL-4R in IL-4-treated cultures (Fig. 6).

Removal of IL-4 from Jijoye Cell Cultures Results in a Transient Up-regulation of the IL-4R—Since the rate of internalization of IL-4 over the 13- and 24-h culture period was similar to the internalization rate measured at the origin, we speculated that the reexpression of the IL-4R was due to an increased receptor reinsertion into the plasma membrane. Thus, cells cultured for 24 h in the presence of IL-4 were washed free of IL-4 and then recultured without IL-4. At various time points, aliquots of cells were harvested, acid-treated, and incubated with IZ5I-IL-4 to determine the total number of IL-4R. Cells preincubated for 24 h with IL-4 display an increased amount (+50%) of IL-4R 2-4 h after the removal of IL-4 (Fig. 7). This increase of IL-4R is only transient since 6 h after the removal of IL-4, cells express approximately the same number of receptors as control cells. The increased binding of IL-4R reflects an increased number of binding sites as determined by Scatchard analysis 2 h after removal of IL-4 (not shown). Removal of IL-4 from cells which had been cultured with IL-4 for 2 h (a time at which cells express the lowest numbers of receptors) results in a rapid increase of cell surface IL-4R 2 h later, but in these conditions, cells never expressed more receptors than control cells.

Biochemical Analysis of the IL-4R: Transient Down-regulation by IL-4—Cross-linking of bound IZ5I-IL-4 to Jijoye cells revealed the presence of three labeled polypeptides with M, 130,000, 80,000, and 70,000 (Fig. 8, lane I). Formation of these complexes was prevented by inclusion of a 10-fold excess of...
unlabeled IL-4, indicating a specific saturable interaction between IL-4 and the binding molecules (Fig. 8, lane 2). Preincubation of cells at 37 °C for 20 min (lane 4) or 90 min (lane 5) with 1 nM unlabeled IL-4, followed by acid treatment and binding/cross-linking of $^{125}$I-IL-4, demonstrated a strong decrease in the radioactivity of the three bands. Cells preincubated for 24 h with unlabeled IL-4 (lane 6) display the same complexes as cells cultured without IL-4 (lane 7). These results show that the three cell surface IL-4 binding proteins disappear and reappear in a coordinated fashion in response to IL-4 exposure.

**Induction of FcεR2/CD23 Requires Prolonged Exposure to IL-4**—Incubation of Jijoye cells in the presence of IL-4 for 48 h results in the cell surface expression of FcεR2/CD23. To determine whether this phenomenon required a short or a prolonged exposure of cells to IL-4, cells were cultured for varying periods of time with IL-4, then recultured without IL-4 so as to reach a total incubation time of 48 h. IL-4 was removed either by centrifugation/washes or by addition of a neutralizing anti-IL-4 rabbit antiserum. Fig. 9 shows that exposure of Jijoye cells to IL-4 for 2-8 h is not enough to induce FcεR2/CD23 48 h later. Exposure of cells to IL-4 for 20 h resulted in a near-maximal induction of FcεR2/CD23. Thus, induction of FcεR2/CD23 on Jijoye cells in response to IL-4 requires a prolonged exposure of cells to the ligand.

**DISCUSSION**

The present study elucidates the fate of IL-4 and of the IL-4R under "physiological conditions" where IL-4 delivers its biological effects (in this case, induction of FcεR2/CD23). It was shown that a fraction of the cell surface receptor-bound $^{125}$I-IL-4 internalized while another fraction dissociated into the culture medium. Internalization and dissociation of surface receptor-bound $^{125}$I-IL-4 were found to be competitive first order events. The rate of internalization ($k_{in} = 8.5 \times 10^{-4}$ s$^{-1}$, $t_{1/2} = 13$ min) was twice as fast as the rate of dissociation ($k_{off} = 4.7 \times 10^{-4}$ s$^{-1}$, $t_{1/2} = 25$ min). Thus, for every three molecules of IL-4 associated to the cell surface receptor, two were internalized while one dissociated. Internalization is likely due to receptor-mediated endocytosis rather than fluid phase pinocytosis since the internalization half-time is of the order of a few min ($t_{1/2} = 8.9$ min) and sodium azide inhibits the appearance of acid-insensitive radioactivity (not shown). Once internalized, $^{125}$I-IL-4 is degraded ($t_{1/2} = 68$ min). Inhibition of $^{125}$I-IL-4 degradation by lysosomotropic agents suggests that $^{125}$I-IL-4 degradation occurs in lysosomes. Thus IL-4 seems to be processed like EGF (22), insulin (23), colony stimulating factor 1 (16), IL-2 (26), and IFN-γ (27–29). Internalization of EGF (30) or insulin (31) is accompanied by a down-regulation of their cell surface receptors, whereas internalization of IFN-γ (28) or bombesin-like peptides (32) is not accompanied by a down-regulation of their respective receptors. Here, it is demonstrated that the IL-4R is rapidly down-regulated from the cell surface with a nadir reached after 2 h. However, upon prolonged incubation of the cells with IL-4, it was observed that the IL-4R were reexpressed on the cell surface. Since the internalization rate of the occupied receptor, measured between 13 and 24 h (0.75 molecule/s), was found to be close to the internalization rate of the unoccupied receptor at the origin (0.84 molecule/s), it is concluded that the IL-4R reexpression seen at 24 h is a consequence of an enhanced reinsertion of the unoccupied receptors into the plasma membrane. This is substantiated by the demonstration that removal of IL-4 from the culture medium at 24 h, which decreases internalization, results in a rapid but transient up-regulation of cell surface receptors (Fig. 7).

The increased reinsertion of the receptor into the plasma membrane may come from three sources: recycling, mobilization of an intracellular pool, and/or accelerated synthesis of the receptors. Addition of chloroquine to cells cultured in the absence of IL-4 delayed the reexpression of the IL-4R thus indicating that receptor recycling may be partially involved in the reexpression of the receptor (25). Preliminary experiments showed that the total number of IL-4R measured after solubilization of the cells was equivalent to the number of receptors measured on the surface of intact cells. This observation suggests that the reexpression of cell surface receptors cannot be accounted for by the presence of a secondary readily accessible intracellular pool of receptors. Finally, the addition of cycloheximide prevented the reexpression of IL-4R, demonstrating that protein synthesis is required and that recycling alone is insufficient to replenish the cell surface pool of IL-4R.

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Regulation of cell surface IL-4R has also been investigated at the molecular level of cross-linking experiments. $^{125}$I-IL-4 was specifically cross-linked to three polypeptides of M, 130,000, 80,000, and 70,000. The M, 130,000 protein probably corresponds to the previously identified M, 139,000 protein (33); however, the 80,000 and 70,000 proteins were not described in this earlier study. These two proteins may either represent degradation products of the M, 130,000 polypeptide or may represent polypeptides in an IL-4R complex. Whatever the relationship between these three proteins, the experiments described here clearly show that they are tightly associated since down-regulation and reexpression of cell surface IL-4R correlated with the coordinated disappearance and reappearance of these three proteins on the cell surface.

Earlier we described that the biological effects of IL-4 on normal human B cells required a prolonged contact between IL-4 and the target cells (e.g. induction of proliferation (3), induction of IgG and IgM production (9), inhibition of IL-2-dependent B cell proliferation (6)). It is comparable to the 8-12-h EGF exposure time of cells requisite for cell commitment and entry into $\text{S}$ phase (34), the continuous presence of IFN-α for the inhibition of Daudi cell growth (35), or the minimum 4-h contact necessary for IFN-γ to induce tumoricidal activity of murine macrophages (27). On the other hand, the Fcε- and In- inducing activity of IFN-γ (29) on macrophages was found to require only short periods of incubation (5-30 min) with the ligand. However, the IL-4-mediated induction of FcεR2/CD23 required a prolonged exposure of the cells to saturating concentrations of IL-4 since 8 h of incubation were totally ineffective. Maximal induction of FcεR2/CD23 required cells to be in contact with IL-4 for 24 h and was thus comparable, in this respect, to the other biological effects of IL-4. We calculate that during this period of time, 10$^6$ cells (960 sites/cell) have internalized and degraded (3.5% degraded over 24-h incubation, see Fig. 5) 44-fold more IL-4 than the original number of IL-4R expressed at the cell surface. Whether the induction of FcεR2/CD23 is directly linked to the amount of internalized and processed IL-4 or to an increase of receptor turnover during the culture period remains to be elucidated.

Biochemical analysis of the reexpressed receptor versus expressed receptor did not reveal any differences in the struc-

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tecture or binding parameters of the receptor. These observations do not favor the possibility that reexpressed IL-4R may have different polypeptide components or may display a very high affinity (Kd > 4 x 10^{-11} M or undissociable [12]-IL-4 binding), as was described with EGF (36) and platelet-derived growth factor (37). Additional studies will be required to elucidate whether the reexpressed IL-4R is really identical to the expressed receptor (i.e. delivery of different biochemical signals).

As a conclusion, the study established that internalization of IL-4 results in the rapid down-regulation of the IL-4R, followed by a reexpression of the receptors on the cell surface. This latter phenomenon may represent an important step for the delivery of IL-4 biological activity.

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The following relationships can be derived from this treatment:

1. The rate of disappearance of cell bound 125I-lL-4 (d[125I-lL-4] / dt) is equal to the sum of the rate of internalization (d[125I-lL-4] / dt) plus the rate of degradation of intact 125I-lL-4 (d[125I-lL-4] / dt).

2. The ratio of cell surface concentration to intracellular concentration (S/C) can be derived.

In other experiments, inhibitors of protein synthesis, receptor recycling and transcription were added during the 37°C preincubation period. 15 min before the addition of 1 nM cold IL-4.

Reproducibility of cell surface receptors on IL-4-pretreated cells.

Jiioye cells (2.5 x 10^6) were incubated at 27°C in RPMI/FCS/Hepes medium (5% CO2 atmosphere) for 60 min. After centrifugation at 1000 g for 5 min, the supernatants were removed, and the cells were incubated at 37°C for 30 min.

Covalent cross-linking and gel electrophoresis.

107 cells/mL were incubated in RPMI 1640, 20 ml hepes, pH 7.3 (RPMI/Hepes) at 4°C in the presence of 5% CO2 at 12.5°C. After 2 h, the cells were centrifuged, washed twice with RPMI/FCS/Hepes medium (100 ml) and resuspended in 2 ml PBS-50 mM Hepes, pH 8.5. Then, a final concentration of 50 ml cross-linker agent RSA was added to the incubation medium. After 30 min, 200 ml of Tris-HCl, pH 8.5 was added and 1 min later a cross-linker agent was added and washed twice with 5 ml PBS pH 7.2. The washing of cells was repeated 3 times. Cells were centrifuged, washed in PBS and resuspended in 2 ml gel electrophoresis buffer (0.05 M Tris-HCl, 0.1 M NaCl, 0.0005% SDS, 0.001% bromophenol blue, 0.1% sodium dodecyl sulfate). The gel was subjected to electrophoresis at 90 V for 4 h.

Analysis of IL-4 receptor (FeRccD32) expression.

Induction by IL-4 of FeRccD32 expression in Jiioye cells was performed as described in (3). Briefly, 3 x 10^6 Jiioye cells were cultured in 24-well plates in culture medium (1 ml culture medium) in the absence (spontaneous expression) or presence of IL-4 (1 nM). After 48 h, cells were harvested, washed and stained according to standard techniques with MoAb 25C and fluorescently-labeled anti-mouse Ig. The FeRccD32 expression was monitored by flow cytometry.
Fate of IL-4 and Its Receptor after Endocytosis

FIG. 6. Effects of agents which block IL-4 receptor recycling (chloroquine), protein synthesis (cycloheximide), or DNA transcription (actinomycin D) on IL-4 binding to the cell surface. (A) CHO cells (1.5 × 10^6 cells/ml at 37°C) were pretreated with 1 mM IL-4 for 15 min before the addition of 1 nM IL-4. The experiments were then carried out exactly as described in Fig. 4A. Chloroquine (CQ) 150 μM or cycloheximide (CHX) 150 μM and actinomycin D (A) (10 μg/ml) were added, or not (o) in the cell culture (1.5 × 10^6 cells/ml at 37°C) 15 min before the addition of 1 nM IL-4. IL-4 binding to the cells was plotted as a percent of 125I-IL-4 binding obtained at the same time point with cells preincubated without IL-4 and drugs. Values represent the means of data (9) obtained in 3 independent experiments ± SEM.

FIG. 7. Regulation of IL-4 receptor after removal of IL-4 from cell cultures. J558 cells (3.5 × 10^5/ml) were precultured at 37°C for 2 h. At the indicated time points, cells were treated with the pit 3-glycine-ME dissociating buffer (1% SDS, 4°C) and the binding of 125I-IL-4 to the cells after 24 h (o) or 4 h (A) or 2 h (A) culture with 1 nM IL-4 (a) was plotted as a percentage of the binding to cells which had been cultured without IL-4 (100%). Values represent the means of 3 independent experiments ± SD.

FIG. 8. Cell surface proteins down regulated by IL-4. J558 cells were precultured with (lanes 3, 4, 5, 6) or without (lanes 1, 2, 7) 1 nM IL-4 for 2h (lanes 1,2,3) 20 min (lane 4), 90 min (lane 5), 24 h (lane 6,7). Then, cells were treated with glycine-NaOH pH 3 buffer and 125I-IL-4 binding to the cells was carried out as described in Experimental Procedures. 1% Triton X-100 cell lysates were assayed by SDS-PAGE (200 μg of protein/lane, 6-15% polyacrylamide gel gradient). Autoradiograms of the dried gels were revealed after 24 h exposure. Non-specific 125I-IL-4 cross-linking has been determined in parallel experiment, where 10 nM IL-4 has been added during 125I-IL-4 binding to the cells (lane 2).

FIG. 9. Induction of FcγR2/CD23 or J558 cells requires prolonged exposure to IL-4. J558 cells were incubated in the presence of 1 nM IL-4. Then at various periods of time, IL-4 was removed from the medium either by washing (Ο) or by adding a neutralizing polyclonal antibody specific for IL-4 (10 μg/ml) (●). FcγR2/CD23 expression was measured by flow cytometry by staining with MoAb 25 and FITC conjugated antimouse Ig after a total 48 h incubation time. Each point represents the mean ± SD of triplicate.