suggest that binding and processing of N-formyl peptides by living human neutrophils may follow the general pathway of receptor-mediated endocytosis described by Willingham & Pastan [15] and Geisow [16] involving binding of ligands to homogeneously distributed surface receptors [3, 4, 6, 7, 9, 10], clustering of receptor–ligand complexes [3, 4, 6, 7, 9, 10], and the passage of an occupied receptor in the form of receptosomes [3, 6, 9] to the perinuclear Golgi region of the cell [3, 4, 7, 9, 10] from where the fluorescent ligand is translocated into intracellular motile vesicular structures [3, 4, 6, 9, 10, 17].

The microscopic observations on binding of TMR-peptide to neutrophils, which were treated with the cytoskeleton-destructing drugs, cytochalasin B or colchicine, give rise to the following conclusions:

1. Neutrophils treated with cytochalasin B before TMR-peptide binding do not polarize and do not show any asymmetrical distribution of ligand–receptor complexes, suggesting that N-formyl-peptide-induced front–tail polarity of neutrophils is associated with asymmetric redistribution of ligand–receptor complexes.

2. Colchicine pretreatment of neutrophils exerts no apparent inhibitory effect on TMR-peptide internalization, suggesting that intact microtubules are not necessary for the removal of receptor–ligand complexes from the cell surface. After the initial step of ligand binding to the cell surface at elevated temperatures, fluorescent TMR-peptides aggregate and accumulate into a cap at the rear end of the neutrophil even in colchicine-pretreated cells. Evidently, aggregation and cap formation of TMR-peptide may proceed independently of an intact microtubular network.

3. The passage of clustered TMR-peptides from the rear end of a polarized neutrophil to the perinuclear region of the neutrophil is blocked by colchicine pretreatment. Tentatively, we conclude from these observations that the passage of TMR-peptide from the rear end to the perinuclear region of the neutrophil depends on an intact microfilament network.

Sullivan et al. [14] used scanning electron microscopy to investigate the binding of a haemocyanin-linked N-formyl peptide to surface receptors on polarized but glutaraldehyde-fixed rabbit peritoneal neutrophils. These authors demonstrated surface binding of this ligand to the front and the mid-section of the cell. In contrast to Sullivan et al. [14], we described the fate of the fluorescent N-formyl peptide TMR-peptide in living polarized human neutrophils in real-time in the presence of excess free ligand. Thereby novel features of the processing of N-formyl peptides by neutrophils were uncovered that may be significant for ligand–receptor processing and cell activation. These findings correlate well with our results obtained biochemically by tracing the receptor in stimulated neutrophils by means of photoaffinity labelling and subcellular fractionation techniques [1, 3, 7].

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The interferon-γ receptor on human monocytes, monocyte-like cell lines and polymorphonuclear leucocytes

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Introduction

Interferon (IFN)-γ is a member of a group of IFN proteins which display an array of biological effects on almost all cells of the immune system. The effects of IFN-γ on monocytes/macrophages can influence cell differentiation, inflammation, host defence and tissue repair [1, 2]. Some of the more specific effects of IFN-γ on these cells include enhancement of Fe receptors [3], increased expression of class II histocompatibility antigens [4], and tumoricidal [5] and microbicidal [6] activity; these effects may occur at very low concentrations of IFN-γ (1–3 pm). In order to develop responsiveness at such low concentrations of ligand, a highly specific receptor system has evolved on macrophages and other cells of the immune system. However, since most of the effector functions of macrophages required 24–72 h for expression, there is only incomplete information on the mechanisms underlying the ability of IFN-γ to induce such effects. Our laboratory has approached the question of signal transduction by initially defining the characteristics of the receptor–ligand interaction and the immediate post-binding effects of this interaction in human monocytes, neutrophils and monocyte-like cell lines.
Binding of IFN-γ to its receptor

In 1985 we [7] and others [8, 9] described the binding of recombinant IFN-γ (rIFN-γ) to its receptor on human monocytes. Escherichia coli-derived rIFN-γ was provided by Genentech, Inc. and Hoffmann-LaRoche, Inc. The molecule was purified to homogeneity as assessed by polyacrylamide gel electrophoresis, contained no carbohydrates, and in its monomeric form had an M₀ of about 17000. The pl of the molecule was very basic, about 8–8.5, and contained a series of basic amino acids [10–15] at the C-terminal end important for bioactivity [10]. In solution, IFN-γ behaved as a dimer, just as the natural molecule [11]. However, since the stoichiometry of binding has not been clearly defined, all the data presented refer to molecules of monomer bound per cell.

rIFN-γ was radiolabelled to high specific activity (200–400 Ci/mmol) with Bolton–Hunter reagent [12]. Biologic activity was measured as assessed by anti-viral assay. Bindability of freshly labelled material was usually at least 70%. For peripheral blood monocytes, we obtained highly purified preparations (>90%) by Ficoll–Hypaque sedimentation followed by countercurrent centrifugal elutriation [13]. Polymorphonuclear leukocytes (PMN) were purified to 95% purity by a combination of Ficoll–Hypaque and Dextran T500 sedimentation [14]. Two human myeloid cell lines were studied: U937 cells, derived from a histiocytic lymphoma, and HL60, derived from a myelomonocytic leukemia.

All four types of cells bound rIFN-γ to approximately the same extent: U937, 3500 molecules per cell [7], HL60 and monocytes, 2000 molecules per cell [7], and PMN, 1000 molecules per cell [15]. The affinity of the ligand for the receptor was high at 5-10⁻¹⁰ M⁻¹. At 37°C binding was complete within 5 min. Binding to all cell types was specific for IFN-γ. Only rIFN-γ or natural IFN-γ competed with the radiolabelled rIFN-γ for binding to the cells. Neither IFN-α nor -β blocked the binding of IFN-γ to the cell. This was consistent with the concept that the receptor for IFN-γ was distinct from those for IFN-α or -β. For monocytes, neither pH changes between 6 and 8 nor the absence of calcium in the binding media affected the binding of rIFN-γ to the cell. Only at very low pH (2.5–3.0) was there dissociation of rIFN-γ from the cell (90–95% of the surface-bound radioactivity).

Internalization and degradation of rIFN-γ

Detailed studies on the internalization of rIFN-γ were undertaken in the U937 cell line [16]. U937 cells were exposed to radiolabelled rIFN-γ at 37°C, and aliquots of cells were removed at various times for determination of intracellular rIFN-γ. Intracellular rIFN-γ was determined by treating cells with pH 2.8 acid buffer (glycine) which removed all surface-bound radioactivity. The radioactivity remaining with the cell was then defined as intracellular or acid resistant. rIFN-γ was continuously taken up into the cell at 37°C such that by 30 min 40–50% of the amount maximally capable of binding to the cell was intracellular. The rate of endocytosis was about 32 molecules/min over the first 30 min of endocytosis at a concentration of rIFN-γ of 150 units/ml. At higher concentrations of ligand, more molecules of rIFN-γ entered the cell. However, when the amount of intracellular rIFN-γ was calculated as a percentage of the total amount bound at the various concentrations, there was little difference between the values (36–50%) over a wide concentration range (25–500 units/ml, 1–20 ng/ml). This suggested that there was not a concentration-dependent movement of molecules into the cell, that is, greater receptor occupancy did not induce a larger fraction of molecules to enter the cell.

Once intracellular, IFN-γ was readily degraded. When cells were pulsed with rIFN-γ for 1 h at 37°C, washed and then cultured for an additional 4 h, degradation of rIFN-γ could be monitored by the appearance of free 125I in the medium. Of the cell-associated rIFN-γ, 55% was intracellular after the 1 h pulse. On further culturing, there was relatively rapid degradation such that by 2 h, 96% of those molecules initially acid resistant were degraded. Of those molecules initially cell associated after the pulse, 68% were internalized and degraded within 4 h. Internalization was inhibited by the microtubule and microfilament inhibitors cytochalasin D and colchicine from 49% to 19% at 30 min. Interestingly, those molecules which did enter the cell in the presence of these inhibitors did not become degraded, suggesting that cytoskeletal structures were required for the transport of internalized rIFN-γ to lysosomes for ultimate degradation. Monensin, a proton pump inhibitor, markedly blocked the degradation of ligand compared with control cells (61% versus 26%). Chloroquine had a similar effect. These data suggested that internalized rIFN-γ was degraded in lysosomes. In PMN, IFN-γ bound to the cell was also quickly internalized, with 50–60% of the ligand becoming intracellular at 30 min of a 37°C incubation [15].

The amount of rIFN-γ internalized could be correlated with cell responsiveness by pulsing cells with various concentrations of rIFN-γ [16]. Pulse exposure of cells to IFN-γ for as little as 15 min resulted in enhanced Fc receptor expression 24 h later, longer exposures increased responsiveness. Concentrations of rIFN-γ that resulted in 20% receptor occupancy induced nearly maximal Fc receptor responsiveness. However, for antiproliferation, not only did cells require longer pulse exposures (1 h), but also required pulses over 3 consecutive days to induce a state of antiproliferation that resulted in a 90% reduction of cell number. Also, only concentrations of rIFN-γ resulting in 50–75% occupancy were effective in inducing cell death. This suggested that responses which required multiple levels of control (proliferation) might not be fully capable of expression until receptors have been occupied at high density and for multiple times.

Modulation of IFN-γ receptors

It has been recognized that the corticosteroid dexamethasone enables monocytes to respond to IFN-γ to a greater extent as measured by Fc receptor induction [17]. One possible mechanism for this enhanced responsiveness was a greater number of IFN-γ receptors. We treated elutriated-purified monocytes with dexamethasone (200 nm) for up to 48 h and then measured IFN-γ binding to the cell [18]. In the presence of dexamethasone, monocytes bound 2500 mol of rIFN-γ per cell in contrast to controls which bound only about 900 molecules without a perceptible change in affinity. Since the control monocytes bound fewer molecules than expected, we examined the kinetics of the enhancing effect of dexamethasone. Cells initially bound about 2000 molecules of rIFN-γ. However, after 8 h of culture, cells now bound only 800 molecules. Those cells in the presence of dexamethasone rapidly increased their receptor number from 800 to 3000 over the next 36 h in culture, whereas untreated cells only increased their binding capacity to 1100 molecules per cell. Protein synthesis was necessary, since the enhancement was completely inhibited by cycloheximide. The observed loss of receptors in culture also occurred with PMN, but to a greater extent and more rapidly. PMN lost 80% of their capacity to bind rIFN-γ at 1 h of culture at 37°C [15]. This rapid loss of receptor was abrogated somewhat by
the presence of interleukin-1 in the media, such that the level of receptors fell only to 50% of control.

It had been reported that when monocytes were cultured for several days an enhancement in IFN-γ receptors occurred [19]. We have investigated whether culture of adherent monocytes in the presence of granulocyte-macrophage colony-stimulating factor or macrophage colony-stimulating factor resulted in modulation of IFN-γ receptors on the cell. When monocytes were cultured with either colony-stimulating factor for 4–6 days, there was a 5-10-fold enhancement in the number of receptors compared with the freshly isolated monocytes. The affinity of the receptor for the ligand was somewhat lower at about 10^5 M⁻¹. The receptor number also increased in cells cultured only in media plus human serum, but not quite to the extent seen with the colony-stimulating factors. Some of the increase in receptor number was probably secondary to an increase in cell size, which occurred as the cells remained in culture for several days. These receptors, however, appeared to be the same receptor as that on freshly isolated cells, since a monoclonal antibody to the receptor [20] blocked binding in both fresh and cultured cells. In addition, the colony-stimulating factor induced receptors retained their specificity such that IFN-α or IFN-β did not compete for binding to the receptor.

When adherent monocytes were allowed to adhere to plastic in the absence of serum, we observed the expression of an even lower affinity receptor for IFN-γ. The adherent cells expressed about 10^10 M⁻¹ that this receptor was different from the high-affinity receptor could be demonstrated in two ways. Cross-linking studies, in which 125I-rIFN-γ was cross-linked to the cell, revealed the receptor-rIFN-γ complex to have an M₅₀ of about 100000, whereas the high-affinity receptor-rIFN-γ complex had an M₅₀ of about 125000. Upon reduction the cross-linked high-affinity receptor increased its Mₛ, slightly, indicative of intramolecular disulphide bonds. With the low-affinity receptor, however, upon reduction the high-Mₛ band disappeared completely and two lower-Mₛ bands at 68 000 and 82 000 were now noted. These data were consistent with multiple subunits for the low-affinity receptor. The high-affinity receptor was known to have one protein chain which binds IFN-γ, as determined by the recent cloning and expression of the receptor [21]. The low-affinity receptor might be important in the activation of cells which require unusually large concentrations of IFN-γ to elicit a biological effect [22].

Regulation of receptor expression

A series of experiments were carried out in U937 cells in order to assess the fate of receptors which have internalized rIFN-γ. We initially investigated the ability of the cell to regenerate receptors after trypsinization. Incubation of cells with trypsin (0.1 mg/ml) for 45 min at 37°C resulted in an 80–90% reduction in surface receptor expression. Re-expression of new receptor was rapid, reaching 80% of control levels within 3 h. This re-expression was completely inhibited both by monensin and cycloheximide. These data suggested that both protein synthesis and intracellular trafficking of newly synthesized receptors was necessary for re-expression to occur after trypsinization.

We next investigated whether the receptor was down-modulated after internalization of ligand. When U937 cells were exposed to rIFN-γ at 37°C for 30 min, about 50% of the ligand was acid resistant or intracellular. If the cells were then allowed to rebind rIFN-γ after the acid stripping, maximal binding of rIFN-γ was noted. When this experiment was performed with 125I- and 35S-labelled rIFN-γ, it was clear that while 50% of the rIFN-γ was intracellular, maximal binding could still be demonstrated to receptors on the cell surface. This was confirmed for up to 3 h of incubation with rIFN-γ. No down-modulation of the receptor could be demonstrated. The ability of the U937 cell to maintain receptor expression after endocytosis of ligand was investigated in a number of ways. Solubilized cells expressed a variable amount of intracellular receptor which averaged out to be about 25% of the amount on the cell surface. There appeared to be some degree of recycling, since treatment of cells with monensin alone for only 15 min resulted in a 40% decrease in receptor expression. These data taken together suggested that the ability of the U937 cell to maintain cell-surface expression during endocytosis of rIFN-γ could be a combination of rapid biosynthesis, recycling of internalized receptor and expression of intracellular receptor.

Conclusions

Human monocytes, macrophages and PMN, in addition to monocyte-like cell lines, all express high-affinity receptors for human IFN-γ. Binding of IFN-γ to this receptor initiates a series of events which ultimately lead to expression of a specific function of the cell. Receptor–ligand internalization occurs rapidly and may be important for development of the biological responses of the cell. U937 cells are capable of maintaining maximal levels of receptor expression in the presence of high concentrations of rIFN-γ, such that down-modulation by ligand is not observed. This may be important in allowing cells to remain responsive to IFN-γ at sites of inflammation.


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