Neutrophils Activated by Granulocyte-Macrophage Colony-Stimulating Factor Express Receptors for Interleukin-3 Which Mediate Class II Expression

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Freshly isolated peripheral blood neutrophils, unlike monocyes and eosinophils, do not bind interleukin-3 (IL-3) or respond to IL-3. We show that neutrophils cultured for 24 hours in granulocyte-macrophage colony-stimulating factor (GM-CSF) express mRNA for the IL-3 receptor (R) subunit, as shown by RNase protection assays, and IL-3R chain protein, as shown by flow cytometric analysis using two different specific monoclonal antibodies. This effect was selective for GM-CSF, because granulocyte colony-stimulating factor, tumor necrosis factor-α, interferon-γ, and IL-1 failed to induce the IL-3 receptor. Saturation binding curves with [35S]IL-3 and Scatchard transformation showed the presence of about 100 high-affinity and 4,000 low-affinity receptors. Because neutrophils have been shown to express human leu-kocyte antigen (HLA)-DR in response to GM-CSF, we examined the possibility that IL-3 could augment HLA-DR expression on GM-CSF–treated cells. We found that neutrophils incubated with 30 ng/mL IL-3 as well as 0.1 ng/mL GM-CSF expressed a mean of 2.1-fold higher levels of HLA-DR than with GM-CSF alone (P < .005), confirming the signalling competence of the newly expressed IL-3R. This increase was seen even at maximal concentrations of GM-CSF and represents the first demonstration that GM-CSF and IL-3 can have an additive effect on mature human cells. The augmentation of HLA-DR by IL-3 was specific because it could be inhibited by a blocking anti–IL-3R antibody. Expression of class II molecules by neutrophils under these conditions may have significance for antigen presentation. These results provide further evidence for the role of GM-CSF as an amplification factor in inflammation by inducing neutrophil responsiveness to IL-3 produced by T cells or mast cells.

MATERIALS AND METHODS
IL-3 (human recombinant) was a kind gift of Genetics Institute (Cambridge, MA) and had a specific activity of 0.17 U/μg. GM-CSF (human recombinant) from two sources was used (gifts were from Dr A. O’Garra [DNAX, Palo Alto, CA] and Genetics Institute) with a specific activity of 50 U/μg. G-CSF (human recombinant) was obtained from Amgen (Thousand Oaks, CA).

MoAbs. MoAb against the IL-3Rα chain (9F5), the GM-CSFRα chain (8G6), and β2 (4F3) were raised and characterized as previously described.10 Purified antibodies (IgG1) were used at a final concentration of 1 μg/mL for staining cells. The control antibody was purified (nonimmune) mouse IgG1 (Becton Dickinson, San Jose, CA). Blocking IL-3Rα antibody 7G3 (IgG2a) was raised and characterized as described.99 Anti–HLA-DR (L243), anti–HLA-DR (B7/21), and anti–HLA-DQ (SPVSL3), which were gifts from Dr H. Spits (Netherlands Cancer Research Institute, Amsterdam, The Netherlands), were purified and conjugated to fluorescein isothiocyanate (FITC) in the department using standard methods.12 The control antibody was FITC-conjugated IgG2a (Dako, Bucks, UK).

Anti–CD16, conjugated to phycocerythrin (PE) or unconjugated, was a mouse IgG2a clone CLB-149 purchased from Eurogenetics.
Neutrophil IL-3 receptors. Anti-CD9 was purchased from the Binding Site (Birmingham, UK).

Neutrophil isolation and cell culture. Heparinized blood from healthy volunteers was diluted 1:1 with RPMI 1640 (Sigma, Poole, UK) and separated by Ficoll/Hyphaque (Nycomed, Oslo, Norway) gradient centrifugation. Red blood cells were lysed in ice-cold isotonic (155 mmol/L) ammonium chloride solution. Purity of preparations was greater than 95% neutrophils as judged by morphologic examination of Wright’s-stained cytocentrifuge preparations. Contaminating cells were mostly eosinophils.

For higher purity, neutrophils were prepared in some experiments by centrifugation of blood over a Percoll (Sigma) solution with a density of 1.082 g/mL (for improved separation from mononuclear cells). The granulocytes were incubated with anti-CD9 antibody (1 μg/mL), which binds to eosinophils and not neutrophils, and then with sheep antimouse-coated magnetic beads (10 beads/eosinophil; Dynabeads; Dynal, Oslo, Norway). The eosinophils bound to the beads were then removed with a magnet. This yielded neutrophil preparations of greater than 98% purity. In other experiments, where indicated, neutrophils were purified using metrizamide (Nycomed, Oslo, Norway) multistep gradients, as described,14 and were also greater than 98% pure.

Neutrophils were cultured in 24-well (10⁶ cells/well) or 96-well (10⁵ cells/well) tissue culture dishes (Nunc, Roskilde, Denmark) in RPMI 1640 (GIBCO, Life Technologies, Paisley, UK) with 5% fetal calf serum (FCS; Sigma), penicillin/streptomycin, and glutamine (GIBCO). TF1 cells12 were maintained in RPMI 1640 medium containing 10% FCS and recombinant GM-CSF at 2 ng/mL.

Flow cytometry. All flow cytometric analysis was performed using an EPICS Profile II cell analyzer (Coulter Electronics Ltd, Luton, UK). Neutrophils were prepared for flow cytometry by washing in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA; Sigma) and 0.1% sodium azide and then incubated with 5% human AB serum (Sigma) or 5% rabbit serum (Sigma) on ice to block Fc receptor-mediated binding of MoAbs. Neutrophils were then incubated with saturating concentrations of MoAb on ice for 20 minutes. Directly stained cells were then either read immediately or fixed in PBS/0.4% formaldehyde/2% glucose/0.02% sodium azide for later reading; indirectly stained cells went through a further incubation with FITC- or PE-conjugated goat antimouse antibody (Dako) diluted in PBS/BSA/azide with 2% goat Ig (Sigma). For dual-staining, cells were initially stained indirectly as described above and then underwent a further blocking step in 5% mouse serum before being incubated with directly conjugated antibody.

Neutrophil preparations labeled indirectly for receptor expression were generally examined after approximately 20 hours of culture and were gated for live cells by forward and side scatter criteria. Neutrophils examined for major histocompatibility complex (MHC) class II expression after approximately 40 hours of culture were dual-stained with CD16 antibody/goat antimouse PE and FITC-conjugated antibodies to HLA-DR, DP, and DQ. HLA expression was determined on cells gated for high CD16 expression.

Results are generally expressed as the mean fluorescence intensity (MFI) of the entire (gated) cell population in linear units (histograms shown have a logarithmic scale). Background (negative control antibody, isotype-matched) fluorescence was subtracted in some of the figures, as indicated in the legends.

Binding of 125I-iodine-labeled IL-3 (125I-IL-3). Recombinant IL-3 was labeled with 125I using the iodine monochloride method.15 Freshly labeled IL-3 was incubated with 2 × 10⁶ neuphylls per point for 3 hours at 22°C in medium containing 0.1% sodium azide (binding medium). Specific binding was established by subtracting the counts per minute (CPM) from parallel samples containing 100-fold excess unlabeled IL-3. The Scatchard transformation14 was derived from a saturation binding curve using 3 × 10⁶ neutrophils (metrizamide preparation, >98% pure) in triplicate per point and 10 pmoi/L to 30 nmoi/L IL-3.

Rnase protection assay. Total cellular RNA was isolated from purified neutrophils using guanidinium thiocyanate.16 A 10 μg sample of total RNA was analyzed by Rnase protection assay, as previously described,16 except that only Rnase A was used (20 μg/mL) and that the glycerophosphate-3-phosphate dehydrogenase (GAPDH) probe used as an internal control was synthesised at a lower specific activity using [α-32P]UTP at 4 Ci/nmol. For probe synthesis, IL-3Ra chain cDNA was subcloned into pGEM2 and GAPDH cDNA into Bluescript KS. The probes protect the following fragments of the mRNA, with enzymes used to linearize the transcription template shown in parentheses: 1119-1280 (Pst I) for IL-3Ra chain and 707-810 (Sty I) for GAPDH. Gels were quantified by ImageQuant analysis (Molecular Dynamics, Menlo Park, CA), with GAPDH being used as an internal control.

RESULTS

Expression of receptors for IL-3 and GM-CSF on resting and activated neutrophils. Neutrophils freshly isolated from healthy donors clearly expressed flow cytometrically detectable levels of GM-CSFIRa (Fig 1A), whereas the profile for IL-3Ra was indistinguishable from that of the control antibody (Fig 1B). β, was also detectable with a profile similar to that of GM-CSFIRa on fresh cells (data not shown).
The average MFI for the IL-3Rα (8 donors) was not significantly different from that of the negative control (Fig 2A). Neutrophils incubated overnight (20 hours) in medium alone remained negative for IL-3Rα and positive for GM-CSFRα and β, (Figs 1C, 1D, and 2B). However, when incubated with 1 ng/mL GM-CSF, upregulation of the IL-3Rα was seen in all subjects (n = 9; Figs 1E, 1F, and 2C). Positive staining for IL-3Rα was seen when highly purified neutrophil preparations (>98%) were used and was also seen on dual-stained CD16^hi cells (Fig 1). A second MoAb directed against a different epitope of the IL-3Rα (7G3)^6 produced equivalent results (data not shown).

GM-CSF incubation resulted in downregulation of its own receptor α chain, which would be consistent with the expected effect of GM-CSF resulting in internalization of receptor. Interestingly, the β, was still detectable after GM-CSF incubation, suggesting that free β might be available to pair with the nascent IL-3Rα to form the high-affinity heterodimeric receptor.

The concentration of GM-CSF required to induce IL-3Rα expression is shown in Fig 3. A slight increase was evident at 0.01 ng/mL of GM-CSF in this experiment, but the increase was more substantial at 0.1 and 1.0 ng/mL. Induction of IL-3Rα by GM-CSF correlated with the downregulation of GM-CSFRα. Incubation of neutrophils with other cytokines including IFN-γ, TNF-α, and IL-1 did not induce flow cytometrically detectable expression of IL-3Rα (data not shown).

**Induction of mRNA for the IL-3Rα in neutrophils.** To investigate the mechanism of modulation of IL-3Rα expression, mRNA was purified from neutrophils incubated for 20 hours in medium alone, 2 ng/mL GM-CSF, or 20 ng/mL G-CSF. mRNA from TF-1 cells was used as a control, because these cells express receptors for both IL-3 and GM-CSF. ^17^ RNase protection assay detected a prominent band for IL-3Rα in the GM-CSF–treated neutrophils, whereas only faint bands were present in unstimulated or G-CSF–treated neutrophils (Fig 4). This was confirmed by quantitation of mRNA; in two experiments, IL-3Rα mRNA was increased fourfold and fivefold by GM-CSF, whereas G-CSF treatment resulted in no increase.

**Binding of ^125^I–IL-3 to neutrophils.** Because flow cytometry demonstrated the presence of both α (low affinity) and β, (converts α chains to high affinity) subunits of the IL-3 receptor on GM-CSF treated neutrophils, studies of the number and affinity of IL-3 receptors were performed. Specific binding (total binding CPM minus nonspecific binding) was not detected in fresh neutrophils from normal healthy donors, however ^125^I–IL-3 bound specifically to neutrophils incubated overnight in GM-CSF, but not G-CSF (data not shown). A saturation binding curve and Scatchard analysis of ^125^I–IL-3 binding to GM-CSF–treated neutrophils (high purity preparations, >98%) indicated both high- and low-affinity receptors (Fig 5). In experiment 1, receptor...
IL-3. To determine whether the expression of IL-3 receptors by GM-CSF-treated neutrophils was accompanied by signalling and functional activation, studies of neutrophil MHC class II molecule expression were performed. Class II molecules were not detected on fresh neutrophils. Purified neutrophils were cultured in medium alone, with 0.1 ng/mL GM-CSF, or with GM-CSF and 30 ng/mL IL-3. HLA-DR (as well as DP and DQ) expression was measured after 2 days of culture at 40 hours. Only those neutrophils that were still expressing high levels of CD16 were analyzed, because these cells have been shown to remain viable and functional, whereas the CD16− cells are in the process of apoptosis.16 This also served to definitely exclude any remaining contaminating eosinophils.

In 9 experiments using separate donors, IL-3 increased HLA-DR expression, relative to GM-CSF alone, by a mean of 2.1-fold (range, 1.4- to 3.1-fold), from a mean MFI of 2.71 ± 0.29 to 4.58 ± 0.85 (P < .05, Student's t-test). Donor variability in induction of class II was noted, as has previously been described.7 A subgroup of donors (3/9) expressed markedly higher levels of HLA-DR; the mean MFI of 4.11 ± 0.08 with GM-CSF alone increased to 8.79 ± 0.08 when IL-3 was also present (P < .005). Neutrophil surface expression of HLA-DP and DQ was not detected by flow cytometry in any donor, although, as a positive control, the antibodies used clearly stained activated monocytes and dendritic cells (data not shown). IL-3 had no effect on neutrophils in the absence of GM-CSF in the majority of donors; however, occasionally small increases in HLA-DR expression were seen (Fig 6 and data not shown). Few cells remained CD16 high in the cultures without GM-CSF, but those present did not express detectable HLA-DR. Survival was equivalent between cultures with GM-CSF alone and those with GM-CSF plus IL-3 (data not shown). Dose titration experiments indicated that 0.1 to 1.0 ng/mL of GM-CSF...
induced optimal expression of HLA-DR, whereas the greatest augmentation of expression by IL-3 was seen at 39 ng/mL (Fig 6). Checkerboard experiments confirmed that higher concentrations of either factor did not lead to further increases (data not shown).

To confirm that IL-3 was indeed the active factor in augmentation of HLA-DR expression in these experiments and that it was acting via the newly expressed IL-3Ra, the anti-IL-3Ra blocking antibody 7G3 was used. When 1 μg/mL of 7G3 was added to the IL-3 and GM-CSF-responsive leukemic cell line MOTE, it blocked its proliferative response to 30 ng/mL of IL-3 (by >95%) but not to GM-CSF. 7G3 did not reduce neutrophil HLA-DR expression in response to GM-CSF alone, but completely blocked the augmented expression seen when IL-3 was also present in the culture (Fig 7). Control antibodies 8G6 (binds to the GM-CSFRα but has no inhibitory effect) and anti-β-GAL (isotype-matched IgG2a, against β-galactosidase) did not block HLA-DR induction (6% and 2% reduction, respectively).

**DISCUSSION**

We have used three separate experimental approaches to show induction of the α chain for the IL-3Rα on neutrophils after their treatment with GM-CSF in overnight culture. Using antibodies specific for each receptor chain, we were able to show the presence and modulation of subunits of the receptors for both GM-CSF and IL-3 by flow cytometry. In addition, RNase protection assays showed the induction of mRNA for IL-3 receptor α chain, and binding studies confirmed the specific binding of radiolabeled IL-3 to both high- and low-affinity sites.

Flow cytometric analysis provides clear evidence of IL-3Ra expression by GM-CSF-activated neutrophils. Each cell is positively identified, first as a polymorphonuclear leukocyte on the basis of size and granularity and second as a viable neutrophil by the expression of high levels of CD16 (because the proportion of nonmononuclear cells was in all preparations <2%, contamination by CD16+ natural killer cells would be negligible). Although this method may be less sensitive for receptor detection than radiolabeled binding assays, it excludes the possibility that receptors on small populations of contaminating cells such as monocytes or eosinophils could confound the results. Two separate IL-3Ra antibodies gave consistent results compared with several different negative controls. The specificity of the antibodies has been confirmed, and the pattern of receptor expression on different leukocyte subpopulations (ie, neutrophils, monocytes, and eosinophils) determined by flow cytometry was consistent with previous studies using radiolabeled ligand (reviewed in Lopez et al; data not shown).

GM-CSF induced a concentration-dependent induction of the IL-3Ra on neutrophils, with a reciprocal concentration-dependent downregulation of the GM-CSFαRα. Crossmodulation of the IL-3Ra by GM-CSF is interesting, because these receptors share a common β, that converts the binding of the respective factor to high affinity and is essential for signal transduction. We found that the β, was still immunologically detectable on the cell surface after GM-CSF treatment. This finding is in accord with the binding data, because high-affinity binding probably represents heterodimeric pairing between the low levels of available free β, and some of the IL-3Ra chains, whereas low-affinity binding is due to the excess free α chain monomers.

The ability of GM-CSF to induce expression of IL-3Ra on the neutrophil surface appears to be unique, because other neutrophil-activating cytokines such as IFN-γ, TNF-α, and IL-1 did not induce flow cytometrically detectable expression (data not shown) and G-CSF did not induce IL-3Ra mRNA. IL-3Ra expression is not an inevitable consequence of prolongation of neutrophil survival in vitro, because neutrophils surviving at 20 hours in unstimulated cultures did not express it and because IFN-γ and G-CSF also effectively prolonged survival (data not shown). This pattern is quite different from that seen in endothelial cells (EC), in which TNF-α and IFN-γ induce IL-3Ra and increase levels of the constitutively expressed β. This implies that a variety of different signalling pathways may be used to activate the IL-3Ra gene and that EC and neutrophils respond differently to signals from the same cytokines.

Because the receptor for IL-3 could be induced on neutrophils, we sought to determine whether it could respond functionally. Previously, IL-3 has consistently been reported not to have any effect on mature neutrophils, adhesion to endothelium, phagocytosis and intracellular killing, adherence to antibody-coated matrices, antibody-dependent cellular cytotoxicity, complement receptor 3 expression, and prolongation of survival. Monocytes but not neutrophils pro-
duced IL-8 in response to IL-3. Because resting cells do not express detectable receptors, these negative results were not surprising. However, neutrophils were recently reported to express MHC class II molecules when treated for 44 hours with IFN-γ, GM-CSF, and, although less consistently and strongly, IL-3. These cultures were supplemented with human serum and G-CSF and indicated that, under certain conditions, IL-3 can have a direct effect on neutrophils.

We studied the effect of IL-3 on receptor-positive neutrophil populations, i.e., having been incubated with GM-CSF for 20 hours. In preliminary studies, we found that GM-CSF alone induced significant expression of HLA-DR after 40 hours of incubation, whereas IL-3 alone, in our hands, induced minimal or no expression. However, when IL-3 was combined with GM-CSF, a significant increase in HLA-DR expression above that produced by GM-CSF alone was seen. This was shown to be dependent on the expression of the IL-3Ra because an antibody to IL-3Ra that blocks binding of IL-3 was able to inhibit this augmentation. Surface expression of HLA-DP and -DQ, which is known to be much lower than -DR on other leukocyte types, was not detected on neutrophils under any conditions, which is in agreement with the results of a previous report.

The significance of the expression of HLA-DR by neutrophils is speculative at this stage. Neutrophils also express intercellular adhesion molecule-1 (ICAM-1), an accessory molecule that acts by adhesion to LFA-1 on the T cell to enhance the interaction between the T-cell receptor and the antigen/MHC complex. However, they do not bear another major costimulatory molecule B7-1, even after culture with stimuli that cause maximal expression of class II (L.G., W.B.S., and C.M.H., unpublished results). Therefore, neutrophil HLA-DR may be important in presentation of antigen to CD4+ T cells that are already activated at inflammatory sites and have less stringent activation requirements or may alternatively induce T-cell nonresponsiveness because of inadequate costimulatory activity.

Because both IL-3 and GM-CSF signal through the same β2 transducing molecule, this is likely to be a limiting factor in their quantitative ability to stimulate cells. In eosinophils and monocytes, treatment with both IL-3 and GM-CSF produces no greater effect than either alone; in fact, these cytokines compete with each other for high-affinity binding, an observation that is explained by the hypothesis that the α subunits of their receptors are present in excess of, and compete for, β2 (reviewed in Lopez et al). Fresh neutrophils bind GM-CSF with high affinity only, implying that there is no free GM-CSFα, i.e., that the β2 is present in at least equal amounts, and may be in excess. This is supported by our finding that apparently free β2 remains on the cell surface after GM-CSF treatment, when GM-CSFα chain is no longer detectable, and that high-affinity binding of IL-3, which requires both α and β chains, can be shown at that time. The ability of IL-3 and GM-CSF to act in synergy in their induction of HLA-DR on neutrophils also implies that the β2 is present in excess and that occupation of both of the respective sets of α chains recruits a greater number of β2 into signal transducing complexes than when either cytokine is used alone. However, it must be noted that the signals from each of these cytokines are transmitted at different times, i.e., although the GM-CSF signal may commence immediately, the IL-3 signal cannot occur until IL-3α begins to appear. It is therefore possible that the IL-3 uses β2 that have been recycled after their internalization as part of a GM-CSFR complex.

These results highlight the role of GM-CSF as an amplification factor in inflammation, because it induces responsiveness of neutrophils to IL-3, which may then lead to further activation. Potential sources of IL-3 at inflammatory sites include activated TH cells, mast cells, and eosinophils. Indeed, IL-3 mRNA has been detected in lymphocytes from allergic rhinitis inflammatory tissues and in some patients with rheumatoid arthritis. Therefore, this pathway is potentially implicated in inflammation of immunologic and allergic origin.

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