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Human peripheral eosinophils express functional interferon-gamma receptors (IFN-γR)

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SUMMARY

In order to determine whether or not IFN-γR is associated with regulatory mechanisms on human eosinophil function, we examined the expression of functional IFN-γR on human peripheral eosinophils. In this study, peripheral blood eosinophils were obtained from seven normal controls and 12 patients (bronchial asthma, $n = 9$, and hypereosinophilic syndrome (HES), $n = 3$), and the purity of eosinophils was $97{\cdot}11 \pm 2.31\%$, $n = 19$. We first showed that anti-IFN-γR α-chain MoAb reacted with all tested eosinophils of both normal controls and patients by flow cytometry analysis. We also showed expression of mRNA for the α-chain of IFN-γR in all purified eosinophils of six individuals. Further, to characterize IFN-γR on eosinophils, we did binding experiments with ^{125}I-IFN-γ on purified peripheral eosinophils. The linear Scatchard plot indicated a single type of high-affinity binding sites (dissociation constant (Kd) $= 3{\cdot}89$–$4{\cdot}95 \times 10^{-10}$ M, numbers of binding sites = 183–233/cell, $n = 3$). To determine whether IFN-γR on eosinophils is functional, we examined surface eosinophilic cationic protein (ECP) and CD69 induction after IFN-γR ligation with recombinant human IFN-γ (rhIFN-γ) on eosinophils by flow cytometry. rhIFN-γ stimulation significantly induced both ECP and CD69 expression on the 2–18 h-cultured eosinophils in a dose-dependent manner. Further, the effects of rhIFN-γ stimulation were significantly blocked by both a neutralizing anti-IFN-γ MoAb and a blocking anti-IFN-γR MoAb. These results suggest that human peripheral eosinophils express functional IFN-γR.

Keywords eosinophils IFN-γ receptor eosinophilic cationic protein CD69

INTRODUCTION

IFN-γ is a cytokine produced by T cells and natural killer (NK) cells that plays a central role in regulating immunological and inflammatory processes [1,2]. IFN-γ mediates its pleiotropic effects on cells through interaction with a species-specific receptor at the target cell surface [3–5]. The human IFN-γ receptor α-chain (hIFN-γRα) cDNA has been cloned from B lymphoblastoid Raji cells and only one 2·3-kb mRNA species was found in different cell lines [6] and has been shown to be a 90-kD transmembrane glycoprotein which binds human IFN-γ with high affinity (Kd $0{\cdot}1$–$6{\cdot}3 \times 10^{-10}$ M) [3,4,7,8].

On the other hand, the significance of activated eosinophils as effecter cells releasing several chemical mediators such as eosinophilic cationic protein (ECP) [9] in allergic inflammation has been recognized [10]. CD69 is a 60-kD homodimer which is constitutively phosphorylated and associated with a guanosine triphosphate (GTP)-binding protein [11,12]. It is a very early activation marker for lymphocytes, particularly T cells [12], and has been reported as an activation marker of eosinophils [13].

Further, the regulatory effects of IFN-γ on the functions and differentiation of eosinophils have also been reported [14–16]. Because IFN-γ has also been shown to have supportive effect on eosinophil survival by inhibition of eosinophil apotosis [17], it is important to show whether or not IFN-γ mediates its regulatory effects on eosinophil activation through interaction with IFN-γR.

In this study, in order to determine whether eosinophils express functional IFN-γR, we examined the expression and character of protein and mRNA of IFN-γR on eosinophils and the induction of ECP and CD69 molecules on eosinophils by IFN-γ–IFN-γR interaction.

MATERIALS AND METHODS

Cytokines and antibodies

Recombinant human IFN-γ (rhIFN-γ), a neutralizing anti-human IFN-γ MoAb (H21 clone, IgG2a) [18–20], and two anti-human

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IFN-γR α-chain MoAbs (non-blocking, GIR-94·5·92 clone IgG2b; and blocking, GIR-208, clone IgG1) [21–25] were obtained from Genzyme (Boston, MA). An anti-human ECP MoAb (EG2 clone, IgG2a) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and was biotinylated. PE-conjugated anti-human CD69 MoAb (L78 clone, IgG1), PE-conjugated mouse IgG1 control, anti-mouse IgG antibody conjugated with FITC, and avidin–FITC were purchased from Becton Dickinson (San Jose, CA). Control mouse IgG were obtained from Cappel Labs (Malvern, PA).

Isolation of human blood eosinophils and culture

Heparinized peripheral venous blood was drawn from three untreated idiopathic hypereosinophilic syndrome (HES) patients (one man, mean age 34·2 years, range 4–69 years, number of eosinophils $82–150\times10^2/\mu l$), nine untreated bronchial asthma (BA) patients (five men, mean age 21·8 years, range 9–37 years, number of eosinophils $5·2–12\times10^2/\mu l$), and seven healthy individuals with no symptoms of allergy (five men, mean age 29·3 years, range 21–40 years, number of eosinophils $0·8–3.5\times10^2/\mu l$). Purification of eosinophils was by a two-stage procedure: first, modification of a previously described method involving a combination of dextran sedimentation and centrifugation through Ficoll–Paque (specific gravity = 1·077 g/ml; Pharmacia Fine Chemicals, Piscataway, NJ) gradients to produce granulocyte fractions (bottom fraction of Ficoll–Paque) containing neutrophils and eosinophils [26]; and second, extraction of the contaminating neutrophils using a MoAb against CD16 (FcγRIII)-conjugated magnetic beads in a negative, direct selection procedure using immunomagnetic beads (Advanced Magnetics, Cambridge, MA) [27].

The expression of IFN-γR on peripheral eosinophils

To determine the expression of IFN-γR protein on eosinophil cell surface, we analysed the reactivity of a non-blocking anti-human IFN-γR MoAb to peripheral eosinophils by a fluorescence-activated cell sorter. In these experiments, eosinophils of all donors were used. Briefly, purified eosinophils ($2–5\times10^5$) were incubated with an anti-IFN-γR MoAb (10 μg/ml) or control mouse IgG (10 μg/ml) at 4°C for 30 min, and were then reacted with an anti-mouse IgG antibody conjugated with FITC at 4°C for 30 min. After washing with Hanks' balanced salt solution (HBSS) containing 1% fetal calf serum (FCS) and 0·1% NaN3, the intensity of the fluorescence of cells was calibrated by FACStar (Becton Dickinson). Flow cytometry acquisition conditions were as follows: detectors amplification voltage: FL1 for FITC detection, 630 V; FL2 for PE detection, 590 V; number of events collected in each acquisition, 5000.

Expression of IFN-γR α-chain mRNA on peripheral eosinophils

To determine the expression of IFN-γR α-chain message on peripheral eosinophils, we examined IFN-γR α-chain mRNA expression of eosinophils by reverse-transcriptase polymerase chain reaction (RT-PCR). In these experiments, eosinophils were purified from six patients. Total cellular RNA was extracted from eosinophils by using a guanidine thiocynanate method RNA extraction kit (SepaGene; Sanko Junyaku, Tokyo, Japan).

For cDNA synthesis, 1 μg of RNA was denatured at 65°C for 5 min, and reverse transcribed in a 20-μl reaction volume containing 1× buffer (50 mM KCl, 10 mM Tris–HCl pH 8·3), 10 mM DTT, 300 U Moloney mouse leukaemia virus (MMLV) reverse transcriptase, dNTPs (0·5 mM each), 1 μl RNAase inhibitor, and 1 μl dT16 primer (50 μM) (Perkin Elmer Cetus, Norwalk, CT), incubated at 42°C for 30 min, heated at 94°C for 5 min, and stored at 4°C. PCR was carried out using 20 μl of the synthesized cDNA mix in 100 μl reaction volume containing 1× buffer, dNTP (0·2 mM each; all Perkin Elmer), 1 μM of 3′ and 5′ primers, and 2·5 U taq DNA polymerase (Perkin Elmer Cetus). Samples were overlaid with 50 μl mineral oil (Perkin Elmer Cetus) and cycled in a Thermal cycler (Perkin Elmer Cetus) for 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C for 35 cycles. Five microlitres of this reaction product were then run on a 1·0% agarose gel.

The β-actin 5′ primer (ATCTGGCACCACACCTTCTACAATGAGCTGCG) and 3′ primer (CGTCATACTCCTGCTTGCTGATCCACATCT) and the IFN-γR α-chain 5′ primer (GAGACGAGCAGGAAGTCGATTATGATCC) and 3′ primer (GGTGGGGGCTTTTATTACGGTTATGAGC) were purchased from Clonetech (Palo Alto, CA) [6].

Chracterization of IFN-γR on eosinophils by binding assay for IFN-γ

Binding of ^{125}I-IFN-γ to IFN-γR on human eosinophils was evaluated by Scatchard plot analysis. Briefly, the eosinophils (10^6/100 μl) were incubated at 4°C for 1 h with the various concentration of ^{125}I-rhIFN-γ (3130 kBq/μg; Du Pont Company, Wilmington, DE) in the presence or absence of unlabelled rhIFN-γ in 100 μl of HBSS buffer containing 10 mg/ml bovine serum albumin (BSA) in a 1·5-ml polypropylene tube. The cells were then spun through a mixture of 80% silicone oil and 20% olive oil to separate receptor-bound ligand. After aspirating the supernate, the tube was cut 3 mm above the cell pellet, and cell-associated ^{125}I-rhIFN-γ was counted using a γ-counter (Pharmacia; model 1270 Rack Gamma II). The value of the radioactivity bound in the presence of an excess of unlabelled ligand was subtracted as non-specific binding. The data of saturation binding experiments were analysed using a modified LIGAND program [28,29].

Inhibition of anti-IFN-γR antibody on the induction of ECP and CD69 expression on cultured eosinophils

To determine whether IFN-γ–IFN-γR interaction exerts a regulatory role in eosinophil surface marker induction *in vitro*, we examined the inhibitory effect of anti-IFN-γR MoAb on the expression of ECP and CD69 on cultured eosinophils by flow cytometric analysis. Culture medium was RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% FCS (GIBCO) and 2 mM L-glutamine. Eosinophils (1×10^6) (the purity of eosinophils was $99·23\pm0·88\%, n=4$) with rhIFN-γ (30–1000 U/ml) were resuspended in the culture medium at 37°C for 2–18 h in humidified 5% CO_2 and 95% air. In inhibition experiments on the ligand, in order to neutralize rhIFN-γ in the culture medium by antibodies, anti-hIFN-γ MoAb (10 μg/ml) or control mouse IgG (10 μg/ml) were first added to the culture medium with rhIFN-γ at 37°C for 1 h, and then eosinophils were added to the medium. In inhibition tests on the receptor, in order to block IFN-γR on eosinophils by antibodies, eosinophils were first incubated with anti-IFN-γR MoAb (10 μg/ml) or control mouse IgG (10 μg/ml) in culture medium at 37°C for 1 h, and then rhIFN-γ was added to the medium.

On flow cytometric analysis, the cultured eosinophils ($2·5–5\times10^5$/sample) were washed with HBSS containing 1% FCS and 0·1% NaN_3, and they were incubated with PE-conjugated anti CD69 MoAb (10 μg/ml) or control mouse IgG (10 μg/ml) at 4°C for 30 min. In ECP expression analysis, washed eosinophils ($2·5–5\times10^5$/sample) were incubated with a biotinylated anti-ECP MoAb (5 μg/ml) or control mouse IgG (10 μg/ml) at 4°C for

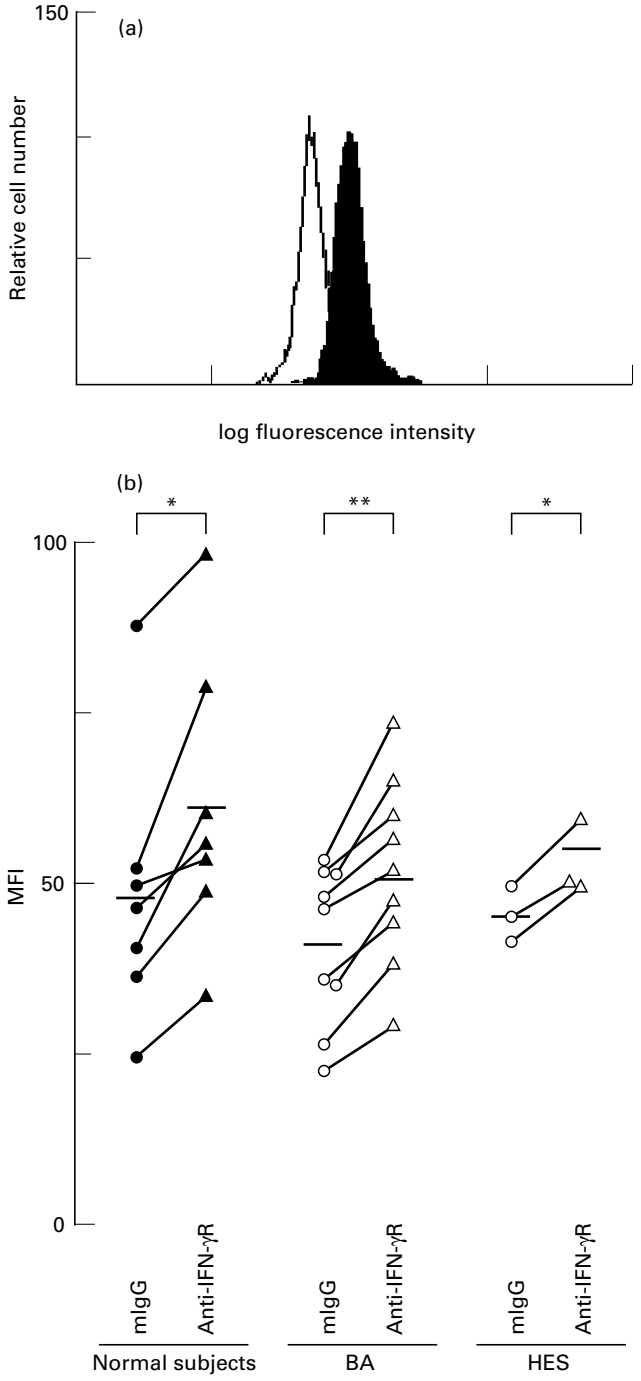

Fig. 1. Flow cytometric analysis of IFN-γR α-chain on peripheral blood eosinophils. (a) Representative data on IFN-γRα expression on eosinophils. The purity of eosinophils of a bronchial asthma patient was 97·56%. (b) Surface expression of IFN-γR α-chain was shown in all peripheral blood eosinophils of seven normal subjects, nine patients with bronchial asthma (BA) and three patients with hypereosinophilic syndrome (HES). The mean fluorescence intensity (MFI) of eosinophils of normal controls was 48·21 ± 19·80 on control mouse IgG study and 61·35 ± 21·17 on anti-IFN-γRα MoAb study ($n = 7$). The MFI of eosinophils of patients with BA was 51·14 ± 13·02 on control mouse IgG study and 41·29 ± 11·58 on anti-IFN-γRα MoAb study ($n = 9$). The MFI of eosinophils of patients with HES was 55·79 ± 4·98 on control mouse IgG study and 45·56 ± 4·10 on anti-IFN-γRα MoAb study ($n = 3$). Significantly different from the mean value of the control IgG: $*P < 0·05$; $**P < 0·01$.

30 min. They were then reacted with avidin-FITC at 4°C for 30 min. The intensity of the fluorescence of cells was calibrated by FACStar (Becton Dickinson).

Statistical analysis

Data are summarized as mean ± s.d. The paired Student's *t*-test was used for statistical comparison of the data, and $P < 0·05$ was considered significant.

RESULTS

Purified peripheral eosinophil conditions

The purity of the examined eosinophil ($n = 19$) was 97·11 ± 2·31% and the viability of the cells was 99·51 ± 0·38%. The contaminated cells were as follows: lymphocytes, 3·53 ± 2·51%; monocytes, 4·05 ± 2·1%; basophils, 0·30 ± 0·44%; and neutrophils, 2·01 ± 2·55%.

Presence of IFN-γR α-chain protein on eosinophils

To determine IFN-γR expression on eosinophils, we first examined flow cytometric analysis of IFN-γR α-chain expression on eosinophils. Flow cytomertric analysis revealed that an anti-IFN-γR MoAb reacted with peripheral eosinophils (Fig. 1a). In order to know the regulatory mechanisms of IFN-γR expression on eosinophils, we analysed the difference in IFN-γR expression on eosinophils between healthy individuals and patients with eosinophilia. IFN-γR expression on eosinophils was shown in all samples examined (Fig. 1b). There was no significant difference, however, in IFN-γR expression on eosinophils between healthy donors and patients. There were also no significant difference in IFN-γR expression on eosinophils between patients with BA and patients with HES (Fig. 1b).

Presence of IFN-γR α-chain mRNA in eosinophils

Further, to certify IFN-γRα expression on eosinophils, we examined IFN-γRα mRNA expression on eosinophils. The total cellular RNA samples from purified eosinophil preparations (purity of eosinophils 99·91 ± 0·08%, $n = 6$) were reverse transcribed into cDNA; cDNA equivalent to 100 ng total cellular RNA per sample was used and regularly yielded bands of the expected size when subjected to PCR with β-actin primers (Fig. 2). This indicated that each sample contained cDNA of comparable quality. We also found strong signals of appropriate sizes of IFN-γRα in purified eosinophil suspensions (Fig. 2).

Scatchard analysis of IFN-γR on eosinophils

To characterize IFN-γR on eosinophils, we conducted binding experiments with ^{125}I-IFN-γ on purified peripheral eosinophils (purity of eosinophils 99·70 ± 0·20%, $n = 3$). A Scatchard analysis is shown in Fig. 3. The linear Scatchard plot indicates a single type of high-affinity binding site. The dissociation constant (Kd) of $3·89–4·95 \times 10^{-10}$ M and receptor density of 183–233 molecules/cell were calculated in the experiments with the LIGAND program.

The induction of CD69 and surface ECP expression by IFN-γ and IFN-γR interaction

Further, to determine the function of IFN-γR on eosinophils, we examined CD69 and ECP induction after IFN-γR ligation with IFN-γ. Because the mean fluorescence intensity (MFI) of eosinophil autofluorescense was very heterogeneous in these experiments

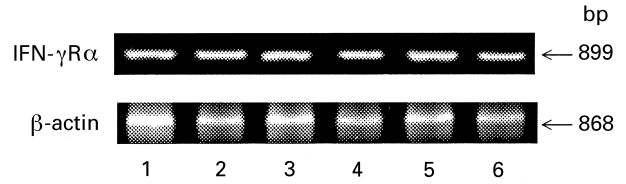

Fig. 2. Demonstration of IFN-γR α-chain mRNA in peripheral blood eosinophils. Oligo(dT)-primed cDNA equivalent to 1 μg total cellular RNA was obtained from purified eosinophils of six eosinophilia patients (purity of eosinophils was 99·91 ± 0·08%). Subsequent polymerase chain reaction (PCR) amplification was carried out for 35 cycles using sequence-specific 5′ and 3′ primers. The reaction product was run on a 1·0% agarose gel.

(MFI = 25·38–87·72), the data on MFI of the surface molecules were shown in ratio form as follows:

$$\text{MFI ratio} = \frac{\text{MFI of anti-CD69 or -ECP MoAb-reacted cells}}{\text{MFI of control mouse IgG-reacted cells}}$$

The MFI ratio of induced and CD69 surface ECP on the cultured eosinophils by rhIFN-γ (100 U/ml) stimulation was increased up to 18 h incubation (data not shown). Further, 30–1000 U/ml of rhIFN-γ stimulation significantly induced CD69 and surface ECP expression on the 18 h-cultured eosinophils in a dose-dependent manner (Fig. 4a,b).

A neutralizing anti-IFN-γ antibody significantly inhibited the expression of CD69 on the 18 h IFN-γ-stimulated eosinophils by 31·27–37·50% ($P < 0·01, n = 4$), and the expression of surface ECP on the 18 h IFN-γ-stimulated eosinophils by 23·30–48·95% ($P < 0·01$, $n = 4$) (Fig. 5a,b). Further, a neutralizing anti-IFN-γR MoAb significantly inhibited the expression of CD69 on the 18 h IFN-γ-stimulated eosinophils by 37·50–65·02% ($P < 0·01, n = 4$), and surface ECP on the 18 h IFN-γ-stimulated eosinophils by 39·20–47·48% ($P < 0·01, n = 4$) (Fig. 5a,b).

DISCUSSION

IFN-γ positively and negatively regulates allergic inflammation [30]. IFN-γ has also been reported to have regulatory effects on eosinophil functions [14,15], differentiation [16], and apoptosis [17]. On the other hand, IFN-γR α-chain (IFN-γR) has been shown to be in monocytes [31], many tissues cells [32], as well as lymphoid cell lines. There is, however, no report on the expression

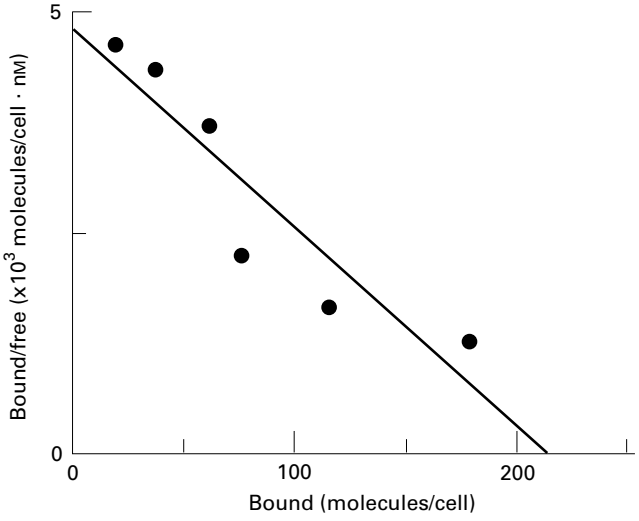

Fig. 3. Scatchard plot analysis of IFN-γ binding to peripheral blood eosinophils. Specific binding of ^{125}I-IFN-γ to eosinophils was determined as described in Materials and Methods. Binding data were analysed using a modified LIGAND program.

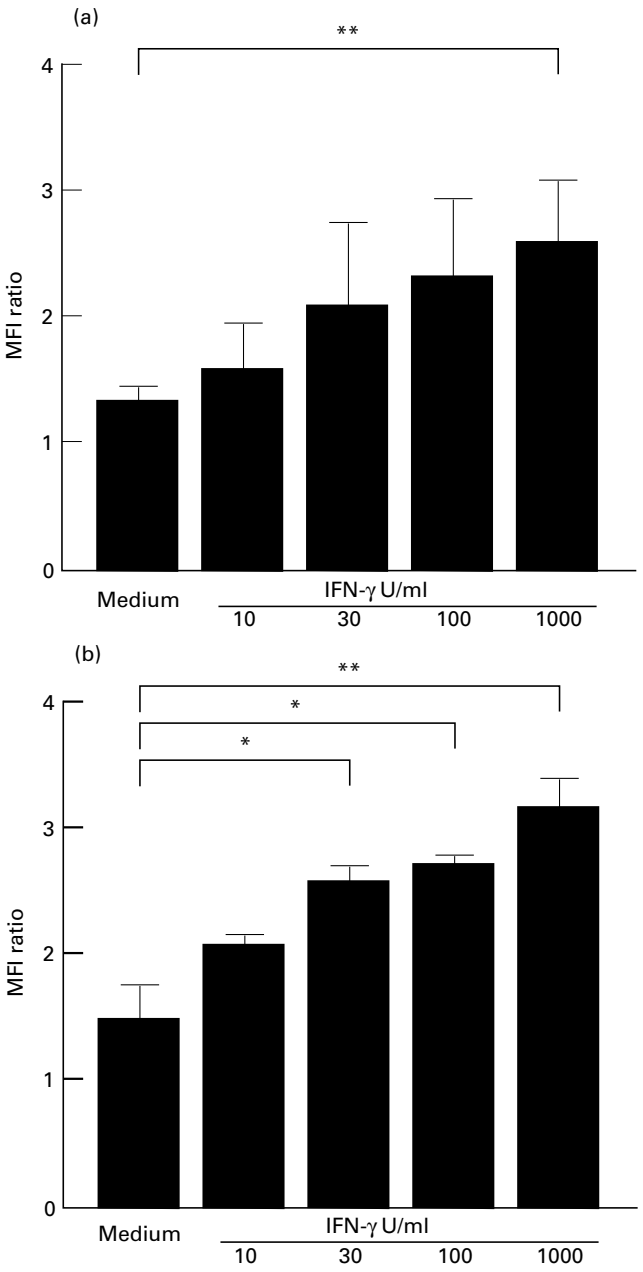

Fig. 4. Effect of concentration of IFN-γ on the expression of CD69 and eosinophilic cationic protein (ECP) on 18 h cultured eosinophils. Eosinophils of four eosinophilia patients were plated at 1×10^6 cells in a 24-well flat-bottomed culture plate at 37°C and rhIFN-γ (30–1000 U/ml) was added to the eosinophil culture from time zero to 18 h. Flow cytometric analysis for expression of (a) CD69 and (b) ECP was then performed. The data on mean fluorescence intensity (MFI) of the surface molecules are shown in ratio form as follows: MFI ratio = MFI of anti-CD69 or -ECP MoAb reacted cells/MFI of control mouse IgG-reacted cells. Data are means ± s.d. for four experiments. Significantly different from the mean value of the control: $*P < 0·05$; $**P < 0·01$.

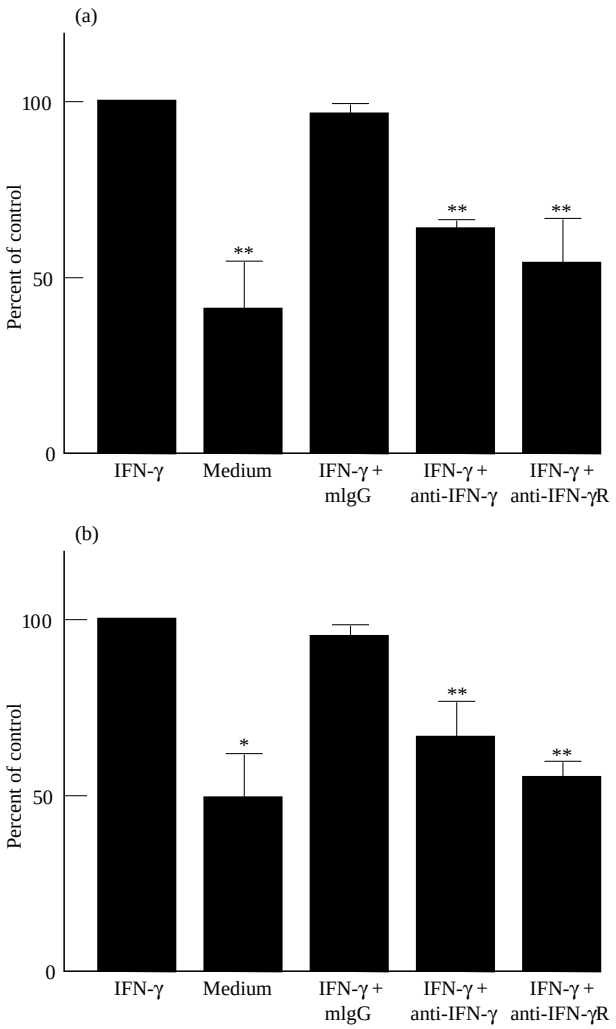

Fig. 5. Analysis on anti-IFN-γR blocking MoAb effects on the induction of eosinophil surface molecules. Effect of anti-IFN-γRα blocking MoAb and anti-IFN-γ neutralizing MoAb on IFN-γ-induced (a) CD69 and (b) eosinophilic cationic protein (ECP) expression on eosinophils.The axis is normalized to the control values on rhIFN-γ (1000 U/ml) stimulation. Data are means ± s.d. for four experiments. Significantly different from the mean value of the control: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

of IFN-γR on human eosinophils. In this study, we first showed the expression of IFN-γR α-chain protein on eosinophils by flow cytometric analysis (Fig. 1) and the expression of IFN-γR α-chain mRNA in eosinophils by RT-PCR (Fig. 2). There was little heterogeneity in the intensity of expression on both IFN-γR protein and mRNA among all samples examined (Figs. 1 and 2). Even in the eosinophils of patients in activated disease stages, the intensity on IFN-γR expression was not significantly higher than the IFN-γR expression intensity of eosinophils of normal control donors. In other experiments, we failed to elucidate the regulatory mechanism of IFN-γR expression on eosinophils by stimulation with several recombinant cytokines, including recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), rhIL-3, rhIL-5, and recombinant human tumour necrosis factor-alpha (rhTNF-α) *in vitro* culture (data not shown).

To characterize further IFN-γR on eosinophils, we also determined the dissociation constant of eosinophils by Scatchard plot analysis (Fig. 3). The Kd of IFN-γR on eosinophils of three patients ($4{\cdot}44 \times 10^{-10}$ M) is almost the same amplitude as on other cells [3,4,7,8], and the density of the receptor was about 200/cell. Because the intensity of IFN-γR expression on eosinophils was not significantly different between patients and normal controls, there might be a regulatory mechanism of the affinity of IFN-γR on eosinophils for IFN-γ. The low density of IFN-γR on eosinophils can also explain the small difference of MFI between anti-IFN-γR-stained and control mouse IgG-stained cells (Figs. 1 and 3).

Furthermore, we showed rhIFN-γ–IFN-γR interaction exerted effects on surface marker induction of peripheral eosinophils in a dose-dependent manner (Fig. 4a,b). CD69 is a very early activation marker for lymphocytes, particularly T cells [12], and has been reported as an activation marker of eosinophils [13]. Hartnell *et al*. have also shown that rhIFN-γ as well as GM-CSF exhibits an effect on CD69 expression of eosinophils [13]. We showed that IFN-γR could transduce the signal by rhIFN-γ stimulation into eosinophils by using specific blocking antibody against IFN-γR α-chain (Fig. 5a). An anti-IFN-γR α-chain MoAb as well as a neutralizing anti-hIFN-γ MoAb significantly blocked the effects of rhIFN-γ on CD69 induction on eosinophils. We also examined the surface induction of ECP on cultured eosinophils. Insofar as the viability of cultured eosinophils was > 95%, rhIFN-γ significantly induced the expression of ECP on cultured eosinophils in a dose-dependent manner (Fig. 4b), and this supportive effect on eosinophil surface ECP induction of rhIFN-γ was also blocked by the anti-IFN-γR α-chain MoAb (Fig. 5b).

In conclusion, we have shown that human peripheral eosinophils express functional IFN-γR.

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