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Macrophage Colony-Stimulating Factor Stimulates Synthesis and Secretion of a Mouse Homolog of a Human IgE-Dependent Histamine-Releasing Factor by Macrophages In Vitro and In Vivo

Shigetada Teshima, Kazuhito Rokutan, Takeshi Nikawa, and Kyoichi Kishi

Treatment of murine resident peritoneal macrophages with macrophage-CSF (M-CSF) up-regulated the synthesis of a discrete set of proteins, including a 26-kDa protein (p26). The sequence of 20 NH₂-terminal amino acids of the purified p26 was identical with the mouse homolog of a human IgE-dependent histamine-releasing factor (HRF). Among macrophage activators tested (M-CSF, granulocyte-macrophage-CSF, IL-3, TNF-α, IFN-γ, and LPS), only M-CSF could up-regulate the p26 HRF synthesis by cultured macrophages. M-CSF not only increased the levels of p26 HRF mRNA and protein, but also stimulated the secretion of an N-glycosylated p26 HRF with a m.w. of 30 kDa. Repeated injections of M-CSF into mouse peritoneal cavity for 4 days elicited macrophages expressing abundant p26 HRF. A single i.p. injection of M-CSF failed to increase the p26 HRF level in peritoneal macrophages of thioglycollate-, LPS-, or adjuvant-treated mice, while M-CSF challenge to OVA-immunized mice caused macrophage infiltration and overproduction of p26 HRF, similarly as did OVA challenge. The Ag-specific priming for enhanced synthesis and secretion of p26 HRF by M-CSF was also demonstrated in cultured macrophages prepared from OVA-immunized mice. An i.p. injection of M-CSF or recombinant p26 HRF triggered eosinophil recruitment, even in the absence of the Ag, in the sensitized mice, but not in normal mice. Furthermore, recombinant p26 HRF could induce eosinophilia without marked macrophage and lymphocyte infiltrations. Our results suggest that p26 HRF secreted by M-CSF-stimulated macrophages may be an important mediator for the late phase allergic inflammation. The Journal of Immunology, 1998, 161: 6356–6366.

In response to cytokines or bacterial products, resident macrophages markedly enhance or newly induce their functions, including chemotaxis, phagocytosis, Ag presentation, and the capacity for microbial or tumor cell killing (reviewed in Refs. 1 and 2). These actions are largely mediated by biologically active products secreted from macrophages, influencing the surrounding cells and macrophages themselves. These products include reactive oxygen metabolites, biologically active lipids, proteases, and cytokines, such as IL-1α/β, IL-6, IFNs, TGF-β, and TNF-α (listed in Ref. 3), all of which are important mediators for inflammatory and immune responses.

Macrophage-CSF (M-CSF) plays a crucial role in the survival, proliferation, and differentiation of mononuclear phagocyte lineages (4). This cytokine also acts as a potent activator for tissue macrophages (5, 6). During the activation of macrophages by M-CSF, a number of proteins have been shown to be synthesized (7–11). We reported that M-CSF induced several heat-shock proteins in murine resident peritoneal macrophages, in association with an increased capacity for superoxide anion (O₂⁻) production (12).

During identification of macrophage cellular proteins whose inductions may be involved in the macrophage activation by M-CSF, we found a unique protein with a molecular mass of 26 kDa (p26). M-CSF up-regulated the synthesis of this protein, and the time course and magnitude of the p26 induction were not affected by the presence of a protein synthesis inhibitor, cycloheximide (CHX). This unique protein was purified and identified as the mouse homolog of the human IgE-dependent histamine-releasing factor (HRF) that was molecularly identified from culture supernatants of the U937 cell line in a recent report (13).

HRFs are defined as products of activated immune cells, and they interact with basophils and mast cells to cause the release of histamine (14). Two types of HRFs have been described: one induces histamine release in a reaction requiring cell surface IgE, and the other operates independently of IgE. The former has been considered to play an important role in the pathogenesis of late phase allergic inflammation by triggering histamine release from basophils in the absence of Ag. The inflammatory late phase reaction initially requires an Ag challenge, but it occurs 3 to 12 h after the immediate reaction in the absence of additional Ag (15). This late phase reaction requires cell surface IgE (15). The IgE-dependent HRFs with several different molecular masses were found in both in vitro and in vivo, such as culture supernatants from human lung macrophages (16) and platelets (17), or fluids of nasal lavages (18) and skin blisters (19) from patients with late phase allergic reactions. Recently, MacDonald et al. first molecularly identified a human IgE-dependent HRF (13). In subsequent studies, they demonstrated that this molecule directly stimulates the release of histamine, IL-4, and IL-13 by basophils from a subpopulation of allergic donors in a reaction requiring a particular...
type of IgE, referred to as IgE$^-$ (13, 20–22). Furthermore, they have shown that, even in the presence of IgE$^-$, the HRF could prime basophils for enhanced responses to subsequent stimuli, such as anti-IgE or specific Ag (21), suggesting its importance in chronic allergic inflammations. The mRNA for this IgE-dependent HRF was detected in T cells, B cells, mononuclear cells, and fibroblasts, but not in mast cells and basophils (13). However, it is still unknown which cell type is mainly responsible for the IgE-dependent HRF production in the late phase reaction, and what is the main regulator of this production is.

We report here that mouse peritoneal macrophages constitutively express an IgE-dependent HRF (referred to here as p26 HRF), and we show that M-CSF, but not other macrophage activators, including granulocyte-macrophage-CSF (GM-CSF), IL-3, TNF-α, IFN-γ, and LPS, acts as a potent stimulus for the synthesis and secretion of p26 HRF by the cells, both in vitro and in vivo.

Materials and Methods

Chemicals and media

Recombinant human carboxyl-terminal-truncated M-CSF encoding the amino acid residues from 3 to 153 of the native M-CSF (truncated rM-CSF) (23) was provided by Otsuka Pharmaceutical (Tokushima, Japan). Murine rM-CSF, GM-CSF, IL-3, TNF-α, and IFN-γ were obtained from R & D Systems (Minneapolis, MN). LPS from Escherichia coli (K-335), CHX, PMA, superoxide dismutase from bovine heart, and chicken OVA (grade V) were purchased from Sigma (St. Louis, MO). DMEM and methionine-free RPMI 1640 were from Life Technologies, (Grand Island, NY). [35S]-protein-labeling mix, containing 77% 1-methionine and $\geq$18% 1-cysteine (sp. act. of 1000–1200 Ci/mmol), was obtained from DuPont/New England Nuclear, Boston, MA. Glutathione-Sepharose beads, PSS-4T-2 plassid, thrombin protease, and ampholines of pH 4.0–6.5, 5.0–8.0, and 3.5–9.5 were from Pharmacia LKB, Biotechnology (Uppsala, Sweden). JM109 competent cells were from TOYOBO, Tokyo, Japan. N-glycosidase F was from Boehringer Mannheim Biochemica (Mannheim, Germany). Monoclonal anti-human IgE-dependent HRF Ab was obtained from Transduction Laboratories (Lexington, KY). A mouse IgE detection kit was purchased from Seikagaku-kogyo (Tokyo, Japan). Two mAbs raised against two different epitopes of mouse IgG1 were from Zymed (San Francisco, CA).

Preparation of peritoneal macrophages and measurement of $O_2^-$

Resident peritoneal macrophages were prepared from untreated control mice in an LPS-free system, as previously described (24). The isolated peritoneal cells were cultured in DMEM without any supplement for 2 h in culture dishes, and nonadherent cells were removed by washing with ice-cold saline. Attached macrophages were cultured overnight in DMEM and used for experiments. Peritoneal macrophages were also prepared from mice treated with one of the macrophage activators (10 ng/ml murine rM-CSF, 20 ng/ml TNF-α, 50 ng/ml rM-CSF, 300 U/ml IFN-γ, 20 ng/ml IL-3, or 10 ng/ml GM-CSF). During the last 2 h of the incubation, cells were metabolically radiolabeled in methionine-free RPMI 1640, supplemented with 50 μM L-[35S]methionine (80 μCi/ml), 0.1% mouse autologous serum, and the activator. Untreated cells were also labeled in the same manner without any activator. In some experiments, cells were pretreated with 5 μg/ml CHX for 30 min before the pulse labeling and incubated for 2 h with L-[35S]methionine in the presence of 0.1% mouse autologous serum and 5 μg/ml CHX. The labeling was terminated by immediately aspirating the medium and washing three times with ice-cold PBS. Soluble proteins were prepared, as previously described (12).

For SDS-PAGE, the soluble proteins were mixed with the same volume of 2× Laemlli’s buffer and boiled for 5 min. An equal amount of protein (15 μg protein) was separated by SDS-PAGE in a 10 to 20% gradient polyacrylamide gel. The gel was stained with Coomassie brilliant blue R-250, dried for autoradiography. For two-dimensional (2-D) PAGE, the soluble proteins (600,000 cpm of $^{35}$S) were dissolved in 20 μl of lysis buffer, consisting of 0.4% ampholine (pH 4.0–6.5), 0.8% ampholine (pH 5.0–8.0), 0.8% ampholine (pH 3.5–9.5), 5% glycerol, 0.5% Nonidet P-40, 2% 2-ME, and 0.001% bromphenol blue. The samples were analyzed by 2-D PAGE, as previously described (12). The gels were treated with DMSO-2.5-diphenylhexaoxole for fluorography, dried, and exposed to X-Omat JB-1 film (Eastman Kodak, Rochester, NY). The radioactivity of labeled proteins precipitated with 5% (v/v) TCA was counted by a liquid scintillation counter.

Microsequencing

The soluble proteins (1 mg of protein) were separated by 2-D PAGE and were electrophoretically transferred to a polyvinylidene difluoride filter (MiniProBlot membrane, Applied Biosystems, Foster City, CA), according to the method of Matsudaira (25). Briefly, a rabbit was immunized with purified milk casein at a concentration of 4% for 3 seconds and incubated for 30 min in 10 mM 3-[cyclohexylaminol]-1-propanesulfonic acid buffer (final pH 11.0) containing 10% methanol. The gel was also incubated in the blotting buffer for 15 min. After electroblotting at a constant electric current of 50 mA at room temperature for 1 to 1.5 h, the transblot was rinsed with ultrapure water for 3 min, immersed in 100% methanol for a few seconds. The transblot was stained with 0.1% Coomassie brilliant blue R-250 in 40% methanol and 1% acetic acid for 10 to 20 s, to visualize the separated proteins, and then it was destained with several changes of 50% methanol, followed by rinsing with ultrapure water. The transblot was air dried, and the visualized spot of p26 was excised and stored at −20°C. Amino-terminal sequences were determined using a gas-phase microsequencer (model 477A/120A; Applied Biosystems).

Immunoblot analysis

Whole cell proteins (30 μg of protein) were separated in 12% SDS-PAGE and transferred to a polyvinylidene difluoride filter. The filter was blocked with purified milk casein at a concentration of 4% for 30 min at room temperature. The filter was incubated for 1 h at room temperature with a 1:4000 dilution of polyclonal Ab against mouse p26 HRF or a mAb, directed against residues 91–107 of human p26 HRF (26). After washing with PBS containing 0.05% Tween-20, bound Abs were detected by an ECL detection system kit (Amersham). The polyclonal Ab was made by immunizing rabbits with the synthetic peptide corresponding to residues 1–20 of mouse p26 HRF (NH₂-MIYRDLISHDELFSDIYKIR-COOH) (27). The resultant serum was further purified by affinity chromatography with the synthetic peptide-conjugated agarose.

Northern blot analysis

A cDNA probe for mouse p26 HRF was obtained by reverse transcription PCR (RT-PCR) with the following primers: sense primer, 5'-AAAAG GATCCATGATATTACCGGGAC-3'; antisense primer, 5'-AAAAA

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Triton X-100, 1 m terminal amino acid sequence as NH₂-GSMIIYRDLI-COOH.

Temperature. The protein products (1 mg of protein/ml) were stored at

of 10 ng/ml murine rM-CSF and 5 μg/ml CHX was added. After incubation for 30 min with CHX, the cells were metabolically labeled for 2 h with 50 μM [35S]methionine in the presence

of 10 ng/ml murine rM-CSF and 5 μg/ml CHX (B, lanes 2–4). Untreated cells (A, lane 1) and CHX-treated cells (B, lane 1) were labeled in the same manner. An equal amount of protein (15 μg of protein/lane) was separated in 10 to 20% gradient polyacrylamide gel. The gel was stained with Coomassie brilliant blue R-250, dried, and exposed to a Kodak X-Omat film. Molecular masses, indicated by arrows, were calculated from the positions of the molecular mass standards (MWSTD) (Bio-Rad) shown on the left. C, The level of p26 was quantified by densitometric analysis. Similar results were obtained in four separate experiments.

GAATTCTTAAACATTTTTTTCAACATTAA-3' (27). A resultant PCR product was confirmed to be a mouse IgE-dependent HRF by cycle sequencing with a DNA sequencer (model ABI 373; Applied Biosystems).

Total RNA was isolated from macrophages with an acid guanidinium thiocyanate-phenol-chloroform mixture (28). Ten micrograms of total RNA were subjected to electrophoresis in a 1% agarose gel containing 0.6 M formaldehyde and transferred to a Hybond N-plus filter (Amersham). After prehybridization, the filter was hybridized for 16 h at 50°C by incubating with the cDNA probe for mouse p26 HRF in the presence of 100 μg/ml heat-denatured salmon sperm DNA and 10% dextran sulfate. The probe was prelabeled with [α-32P]dCTP to a specific activity of >1 × 10^8 cpm by a random primer kit (Amersham). The filter was washed twice with 2× SSC containing 0.1% SDS at 50°C, and then three times with 0.2× SSC containing 0.1% SDS at 60°C. Bound probes were autoradiographed by exposing the filter to Kodak X-Omat films for 2 days at ~80°C.

Expression and purification of recombinant p26 (rp26) HRF

The resultant RT-PCR product was restricted with BamHI and EcoRI, ligated into the pGEX-4T-2 plasmid, and transfected into JM109 competent cells. After incubation of the cells with 0.25 mM isopropyl-1-thio-

galactopyranoside for 5 h at 25°C, they were lysed in PBS, containing 0.1% Triton X-100, 1 μg/ml aprotinin, and 0.33 mM leupeptin, and they were incubated with glutathione-Sepharose beads for 30 min at 4°C. After washing with PBS, bound glutathione S-transferase fusion proteins were enzymatically cleaved by treatment with thrombin in 50 mM Tris-HCl buffer (pH 8.4), containing 150 mM NaCl and 2.5 mM CaCl2, for 4 h at room temperature. The protein products (1 mg of protein/ml) were stored at ~85°C with 1 μg/ml aprotinin and 0.33 mM leupeptin. This recombinant protein was confirmed to be rp26 HRF by determining the partial NH₂-terminal amino acid sequence as NH₂-GSMIIYRDLI-COOH.

Treatment of macrophage proteins with N-glycosidase F

Whole cell proteins (40 μg of protein), prepared from OVA-elicited macrophages, were dissolved in 0.2 M sodium phosphate buffer (pH 8.6), containing 50 mM 2-ME, 0.2% SDS, 1 μg/ml aprotinin, and 10 μM leupeptin, and they were boiled for 5 min. After addition of Nonidet P-40 at a final concentration of 2.0%, the sample was treated with N-glycosidase F (0.2 or 0.5 U per 40 μg protein) for 15 h at 37°C, and then it was subjected to immunoblot analysis with a mAb against rp26 HRF, as described above.

Treatment of mice with M-CSF, thioglycollate, LPS, or OVA

Male C3H/He-slc mice at 8 to 12 wk of age were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). M-CSF-elicited macrophages were harvested from mice injected i.p. with 25 μg of sterile truncated rM-CSF/mouse every 12 h for 4 days. Control mice were injected with sterile saline in the same manner. LPS- or thioglycollate-elicited macrophages were collected on day 3 after an i.p. injection of 20 μg LPS in saline, or 1 ml of 3% thioglycollate broth, per animal, respectively. For sensitization of mice with OVA, they received an i.p. injection of chicken OVA using aluminum hydroxide to favor specific IgE response (29). In brief, 1 mg of OVA in 1 ml of distilled water was mixed with 1 ml of 20% aluminum hydroxide, and then two drops of 0.02% phenol red was added. The pH of the mixture was adjusted to 7.0. The resulting precipitates of OVA and aluminum hydroxide were washed twice with sterile saline and resuspended in sterile saline at a total volume of 10 ml. One hundred microliters of this stock Ag solution, which contained 10 μg of OVA and 2 mg of aluminum hydroxide, was i.p. injected. Control mice were injected with the material prepared in the same manner without OVA. In a separate experiment, normal mice and the OVA-immunized mice were injected i.p. with 0.2 ml of sterile saline or the same volume of saline containing 2 μg of OVA (30), 25 μg of truncated rM-CSF, or 2 μg of rp26 HRF. Peritoneal cells were collected at the indicated times, and differential cell counts were determined, as described above.

Measurement of serum IgE and IgG1 levels

Ninety-six-well microtiter plates were coated with a mAb against mouse IgE or mouse IgG1. To quantify total serum IgE and IgG1 levels, serum and standard dilutions were incubated overnight at 4°C. After washing with PBS containing 0.02% Tween-20, the bound IgE or IgG1 was detected using horseshadish peroxidase-conjugated mAb against mouse IgE or mouse IgG1, respectively, and tetramethyl benzoin and hydrogen peroxide as substrates. The OD was measured at 450 nm on a microplate reader (ImmunoMini NJ-2300, Inter Med). Titers were determined at the points on the linear part of the titration curve.

Results

Effect of CHX on M-CSF-stimulated protein synthesis in macrophages

In this study, we focused on a 26-kDa protein (p26) whose synthesis was rapidly up-regulated within 4 h after exposure to 10 ng/ml of murine rM-CSF (Fig. 1A). We had already recognized that macrophages contained several CHX-resistant proteins, including a protein with the same molecular mass (24). As shown in

![FIGURE 1](http://www.jimmunol.org/...
Effect of M-CSF on the accumulation of p26 HRF mRNA and protein

Mouse p26 HRF transcripts were amplified by RT-PCR with the primer set of 5'-AAAAGATCTCATATGATCCGGG GAC-3' and 5'-AAAAGATCTTAACATTTTCACCTACCTACAA-3' (27). This amplification product was isolated and cloned. DNA sequencing revealed that the resultant PCR product was comprised of the open reading frame (516 bp) of the mouse translationally controlled tumor protein (27). Northern hybridization with this cDNA probe demonstrated that resident macrophages in culture constitutively expressed the p26 HRF mRNA (Fig. 3A, lanes 1 and 2). The mRNA level increased within 2 h, reaching a maximum at 6 h after treatment with M-CSF (lanes 3–6).

The rabbit polyclonal Ab against residues 1–20 of mouse p26 HRF recognized a single macrophage protein with a m.w. of 26 kDa (Fig. 3B, lane 7) and a p26 HRF (lane 12). The recombinant protein migrated more slowly than native p26 HRF, because the protein had two excess amino acid residues, Gly-Ser, at its NH₂-terminus, derived from expression plasmid. Western blot analysis also shows that murine rM-CSF increased the level of p26 HRF in macrophages (lanes 8–11).

We quantified the amount of p26 HRF by densitometric analysis, using the rp26 HRF as a standard, and estimated that resident macrophages contained 20–30 ng of p26 HRF/10⁶ cells. Treatment with M-CSF for 8 h increased this level about threefold.

Effects of M-CSF on secretion of p26 HRF from cultured resident macrophages

The human IgE-dependent HRF is a secretory protein identified from culture supernatant of U937 cells (13), and it was reported to be present in biologic fluids from patients with chronic allergic inflammations (31). We also tested whether murine rM-CSF could stimulate the secretion of p26 HRF into culture medium. When medium from a 4-h culture of untreated macrophages (5 × 10⁶ cells) was subjected to immunoblot analysis with a polyclonal Ab against 20 NH₂-terminal amino acids of p26 HRF, this Ab recognized a strongly immunoreactive band with a m.w. of 30 kDa (Fig. 4, lane 5), but it did not recognize any protein with a m.w. of 26 kDa. After addition of M-CSF, the secretion of the 30-kDa protein into the medium continued to increase during the experimental period (Fig. 4, lanes 6–8). We also used a mAb against residues 91–107 of human IgE-dependent HRF, which differs from mouse sequence by two amino acids. This Ab also recognized this 30-kDa protein (Fig. 4, lane 9), suggesting that the immunoreactive 30-kDa protein was most likely to be a secretory form of p26 HRF that might be posttranslationally modified, i.e., glycosylation. Densitometric analysis with a rp26 HRF as a standard estimated that cultured resident macrophages (1 × 10⁶ cells) secreted 5–15 ng of the 30-kDa protein per hour into culture medium.

Effect of macrophage activators on p26 HRF induction

Resident macrophages, isolated and cultured overnight under LPS-free conditions, released 78 ± 8 nmol of superoxide anion (O₂⁻)/mg protein/hour (mean ± SD, n = 12) after stimulation by 0.5 μg/ml PMA. These cells were treated for 8 h with 10 ng/ml M-CSF, 20 ng/ml IL-3, 20 ng/ml TNF-α, 300 U/ml IFN-γ, 50
After addition of TNF-α, LPS, IFN-γ, IL-3, or GM-CSF, changes in the p26 HRF level were examined up to 16 h by immunoblot analysis with a polyclonal Ab against murine p26 HRF (Fig. 5B). None of these factors changed the p26 HRF level, again suggesting that the production of macrophage p26 HRF might be up-regulated specifically by M-CSF.

We paid special attention to the effect of GM-CSF on p26 HRF induction, since overexpression of this cytokine has been suggested to participate in allergen-induced late phase reactions in atopic subjects (32–35). Therefore, we performed Northern blot analysis and confirmed that GM-CSF did not increase the p26 HRF mRNA level (Fig. 5C).

Expression of p26 HRF in M-CSF-, LPS-, thioglycollate-, or OVA-elicted macrophages

We also tested whether M-CSF similarly acted as a potent up-regulator of p26 HRF production by macrophages in vivo. A human C-terminal truncated rM-CSF (23) was used in experiments in vivo. Mice were treated with various concentrations of this rM-CSF every 12 h for 4 days, and elicitation of peritoneal macrophages was monitored. We determined that 25 μg of this rM-CSF per mouse was a minimum effective dose in our experimental conditions. When peritoneal cells were collected 8 h after a single injection of truncated rM-CSF (25 μg/mouse) into peritoneal cavity, M-CSF did not increase the peritoneal cell number and p26 HRF levels in macrophages (Fig. 6A, lane 2). However, when the injection was repeated every 12 h for 4 days, the numbers of peritoneal cells and macrophages increased 2.4- and 1.9-fold, respectively. The M-CSF-elicted macrophages contained higher concentrations of p26 HRF (lane 3), compared with those in cells from saline-injected mice (lane 1).

The p26 HRF has been suggested to participate in chronic allergic reactions (31, 36, 37); therefore, it was particularly important to study the effects of M-CSF on macrophages from Ag-sensitized mice. Mice were immunized by i.p. injection of OVA plus
Methods

Arrows indicate p26 HRF.

Fig. 3. Similar results were obtained in four separate experiments.

10 ng/ml GM-CSF for the indicated hours. Northern blot analysis with a cDNA probe for murine p26 HRF was performed, as described in the legend to B. IL-3, or 10 ng/ml GM-CSF, for the indicated hours. Whole cell proteins were extracted and subjected to Western blot analysis with a polyclonal Ab against p26 HRF, as described in the legend to Fig. 3B. Arrows indicate p26 HRF.

C. Total RNA was extracted from untreated cells (lane c) or cells treated with 10 ng/ml GM-CSF for the indicated hours. Northern blot analysis with a cDNA probe for murine p26 HRF was performed, as described in the legend to Fig. 3A. Similar results were obtained in four separate experiments.

Immunization with OVA increased the p26 HRF level in peritoneal macrophages about 3- and 2-fold, respectively. The p26 HRF content in adjuvant- (Fig. 6, lane 7) and OVA-elicited macrophages (lane 10), as well as LPS- (lane 8) or thioglycollate-elicited macrophages (lane 6), was measured by immunoblot analysis. As indicated by an arrow in Fig. 6A, M-CSF-, LPS-, thioglycollate-, adjuvant-, and OVA-elicited cells contained immunoreactive bands with m.w. around 30 kDa, in addition to 26-kDa HRF. These 30-kDa proteins were considered to be posttranslationally modified p26 HRFs, as described in a following experiment. The p26 HRF level was quantified by densitometric analysis, and the results are shown in Fig. 6B.

Immunization with OVA increased the p26 HRF level in peritoneal macrophages; however, adjuvant, LPS, and thioglycollate also increased the level, similar to the effect of OVA (Fig. 6, A and B), suggesting that any macrophage-eliciting agent might nonspecifically up-regulate the p26 HRF synthesis. However, as shown in Fig. 6, A and B, macrophages from OVA-treated mice exhibited a strikingly different response to M-CSF in vivo: a single injection of M-CSF markedly increased p26 HRF in macrophages of OVA-sensitized mice, suggesting that the immunization might prime macrophages for an enhanced response to M-CSF.

We also assessed the level of p26 HRF secreted into peritoneal cavity. For this purpose, peritoneal lavage fluid was concentrated and subjected to immunoblot analysis (Fig. 6C). The polyclonal Ab again recognized a single immunoreactive protein with a m.w. of 30 kDa, as detected in the culture medium (Fig. 4). The mAb against p26 HRF also recognized the same band (data not shown). Treatment with LPS (Fig. 6C, lane 3), thioglycollate (lane 6), adjuvant (lane 8), and OVA (lane 10), as well as repetitive treatment with M-CSF (lane 5), increased the amount of the 30-kDa protein in peritoneal fluid. A single M-CSF injection markedly increased the 30-kDa protein content in peritoneal fluid of an OVA-sensitized mouse (lane 11), while it did not in LPS- (lane 4), thioglycollate- (lane 7), and adjuvant-treated animals (lane 9). Each result was confirmed in four animals, and the results are summarized in Fig. 6D. We calculated, by densitometric analysis, the amounts of secreted p26 HRF into the peritoneal cavity of sensitized animals after M-CSF challenge (Fig. 6C) as about 2 μg per peritoneal cavity.

Effects of M-CSF on macrophages isolated from OVA-immunized mice

The experiments with OVA-immunized mice suggested that sensitization with OVA might prime macrophages for enhanced production of p26 HRF stimulated by M-CSF. To address this issue, peritoneal macrophages were isolated from OVA-immunized mice and exposed to M-CSF in vitro. The induction and secretion of p26 HRF by OVA-elicited macrophages were compared with those by resident cells (Fig. 7, B and D). Resident macrophages started to increase the intracellular content and secretion of p26 HRF 4 h after the addition of murine rM-CSF, and the accumulation and secretion gradually increased during the experimental period. In contrast, the effects of murine rM-CSF on OVA-elicited macrophages were strikingly different: M-CSF accelerated the p26 HRF induction in OVA-elicited cells with a peak at 2 h (Fig. 7A, lane 3), and then declined. As shown in Fig. 7, C and D, M-CSF markedly increased the p26 HRF secretion during the initial 4-h incubation, and about twofold higher concentrations of p26 HRF were recovered in the culture medium of OVA-elicited cells at each time point, compared with those of resident cells (Fig. 7D). Similar
results were observed in experiments with the truncated rM-CSF (data not shown).

As shown in Figs. 6A and 7A, OVA-elicited macrophages contained one or two immunoreactive 30-kDa proteins 2 h after stimulation by M-CSF, which were scarcely detected in resident macrophages, even after treatment with M-CSF (Fig. 3B). The 30-kDa proteins were also recognized by the anti-p26 HRF mAb (data not shown). We considered that these 30-kDa proteins might be an N-glycosylated p26 HRF, since p26 HRF has a potential N-linked glycosylation site (Asp-Xaa-Ser), corresponding to amino acids 51–53 (27). To examine the possibility, whole cell extracts of macrophages from OVA-sensitized mice were treated with N-glycosidase F to remove N-linked oligosaccharides. This treatment decreased the intensities of the 30-kDa proteins by increasing the concentration of the enzyme, and consequently, the amount of p26 HRF was increased (Fig. 8), suggesting that p26 HRF may be secreted after undergoing posttranslational modifications, including N-glycosylation.

Effect of Ag challenge on p26 HRF production in OVA-sensitized mice

The OVA-immunized mice were challenged by an i.p. injection of OVA. The Ag increased the level of the 30-kDa p26 HRF and stimulated its secretion into the cavity within 4 h (Fig. 9, A and B). Abundant p26 HRF continued to be recovered from the cavity until 16 h. As shown in Fig. 9, M-CSF challenge could stimulate the production and secretion of p26 HRF, similar to the effect of OVA. In this case, however, the up-regulation was observed within 2 h, suggesting an important role of M-CSF in the overproduction of p26 HRF in Ag-sensitized mice.

Effect of rp26 HRF on eosinophil recruitment into the peritoneal cavity of immunized mice

Finally, we examined whether rp26 HRF could trigger allergic inflammation. Nonimmunized mice were treated with saline, 25 μg of truncated rM-CSF, or 2 μg of rp26 HRF (Fig. 10, A, C, E, G, I, and K). M-CSF did not cause any cell infiltrations. An i.p. injection of rp26 HRF significantly caused neutrophil accumulation (I), while other cells, including eosinophils, did not infiltrate.

The OVA-immunized mice were also challenged i.p. with 2 μg OVA without adjuvant (OVA + OVA), 25 μg of truncated rM-CSF (OVA + M-CSF), or 2 μg of rp26 HRF (OVA + rp26 HRF), and migration of inflammatory cells was monitored (Fig. 10, B, D, F, H, J, and L). In this case, the immunized mice, injected with saline (OVA + saline), were used as controls. An i.p. challenge with OVA caused acute neutrophil migration (Fig. 10J), followed by lymphocyte (Fig. 10F), macrophage (Fig. 10D), and eosinophil (Fig. 10B) accumulations. We confirmed that M-CSF could promote infiltration of these inflammatory cells, even in the absence of the Ag: M-CSF induced neutrophil infiltration during the initial
4-h period in the OVA-sensitized mice (Fig. 10J), and macrophages (Fig. 10D) and lymphocytes (Fig. 10F) started to accumulate at 8 h. It was particularly important that an i.p. injection of M-CSF or rp26 HRF triggered eosinophil recruitment in the absence of Ag in OVA-sensitized mice (Fig. 10B), but not in non-immunized mice (Fig. 10A). Significant eosinophilia was observed without marked macrophage and lymphocyte infiltrations at 4 h after treatment with rp26 HRF, while OVA- or M-CSF-induced eosinophilia started at 8 h (Fig. 10B), indicating the important role of p26 HRF in facilitating eosinophil recruitment in the Ag-sensitized mice. The OVA challenge appeared to stimulate multiple pathways, including mast cell-dependent processes (Fig. 10J); thereby, it caused more severe eosinophilia than that with M-CSF or rp26 HRF (Fig. 10B). We injected 2 μg of rp26 HRF for these experiments, since the amount of secreted p26 HRF into the peritoneal cavity of the sensitized animals after M-CSF challenge was calculated to be about 2 μg per peritoneal cavity.

**Discussion**

During the activation by M-CSF, macrophages induced a unique 26-kDa protein, whose synthesis was up-regulated even in the presence of CHX. We were particularly interested in this protein, since among macrophage-activating factors tested (LPS, M-CSF, GM-CSF, IL-3, IFN-γ, and TNF-α), only M-CSF could induce p26 in cultured macrophages. These findings led us to consider that M-CSF might induce a novel macrophage function through up-regulating the synthesis of p26. We identified p26 at first as a mouse translationally controlled tumor protein (27), which was originally identified in erythroleukemia cells, MEL cell line C7D. A human homolog was then cloned (26). However, no apparent function of these tumor proteins had been ascribed. During our efforts to elucidate the role of p26 in macrophage functions, MacDonald et al. (13) identified the tumor proteins as a member of IgE-dependent HRFs that trigger histamine release from human basophils in the presence of cell surface IgE. They demonstrated that human p26 HRF activated basophils and eosinophils from allergic donors (13, 20–22, 36), and it was found in late phase skin blister fluids, but not in the early blister fluids (31), suggesting that...
p26 HRF plays an important role in perpetuating late phase allergic reaction.

The acute allergic reaction in allergic subjects by Ag challenge is frequently followed by a recrudescence of symptoms, termed the late phase reaction, which resembles chronic allergic inflammation. The development of allergic disorders has been suggested to depend in part on the activation of eosinophils and basophils, as well as a Th2 lymphocyte subset that elaborates Th2 profile of cytokines at the site of inflammation (38). IL-4 is an essential requirement for IgE production (39). IL-3, IL-5, and GM-CSF promote terminal differentiation of eosinophil precursors, eosinophil activation, and increased survival (40–42). The mRNA expressions of these genes, which are clustered on human chromosome 5, have frequently been documented in allergen-induced late phase reactions in atopic subjects (34, 35, 38), and these cytokines have been suggested to be responsible for allergic tissue eosinophilia.

There is growing evidence suggesting that basophils play more important roles as effector cells in human late phase reactions rather than mast cells, since the elevation of histamine in late phase reactions is not accompanied by the production of prostaglandin D2, a mast cell product (43). In order to reveal the mechanism of basophil activation, over the years, multiple species of HRF that activate basophils were identified (listed in Ref. 14). The IgE-dependent HRF has been considered to be more relevant to late phase allergic inflammations than the IgE-independent HRF. However, most of the HRFs, including cytokines and chemokines, operate independently of IgE. Only p26 HRF was molecularly identified as an IgE-dependent basophil activator in recent reports (13, 20–22).

FIGURE 10. Effects of OVA, M-CSF, and rp26 HRF on cell migration into the peritoneal cavity. Nonimmunized mice (A, C, E, G, I, and K) were given an i.p. injection of 200 μl saline (indicated as “saline”), 25 μg of truncated rM-CSF in 200 μl saline (M-CSF), or 2 μg of rp26 HRF in 200 μl saline (rp26 HRF). The OVA-immunized mice (B, D, F, H, J, and L) were challenged i.p. with 2 μg of OVA without adjuvant in 200 μl saline (OVA + saline), 25 μg of truncated rM-CSF in 200 μl saline (OVA + M-CSF), or 2 μg of rp26 HRF in 200 μl saline (OVA + rp26 HRF). Peritoneal cells were prepared at the indicated times, and differential counts of the exudate cells were determined microscopically after May-Grunwald-Giemsa and nonspecific esterase stainings. Nonimmunized (saline) and OVA-immunized mice (OVA + saline), injected with 200 μl saline, were used as respective controls. The numbers of eosinophils (A and B), macrophages (C and D), lymphocytes (E and F), mast cells (G and H), neutrophils (I and J), and total exudate cells (K and L) are expressed as mean ± SD per animal (n = 4). # Significantly increased vs saline-treated mice (p < 0.01 by Student’s t test). * Significantly increased vs saline-treated mice (p < 0.05 by Student’s t test).
(reviewed in Refs. 36, 37). Both recombinant and native human p26 HRF caused histamine release by basophils from a subpopulation of allergic donors (13). In addition to causing histamine release from basophils in the presence of IgE\(^+\), the recombinant HRF could stimulate the production of IL-4 (20–22) and IL-13 (21) by the cells. Furthermore, the HRF also acted on eosinophils of the patients and caused chemotaxis, calcium flux, and IL-8 production (36, 37), suggesting that p26 HRF plays a key role in the pathogenesis of human allergic diseases. However, it is still unknown which cell type mainly participates in p26 HRF production during late phase reactions, and what the main regulator for this production is. Several important functions of this HRF as a possible participant in human allergic inflammations were reported, while no information is available on the exact roles of this factor in mice. Basophils do not infiltrate into peripheral tissues in mice, and it is unknown whether this factor IgE-dependently triggers inflammatory reactions of mouse cells, as it does in human cells.

Macrophages are one of the major populations of infiltrated cells during the late phase reaction (44, 45), and activated macrophages secrete a variety of biologically active products that play critical roles in chronic inflammation and immune responses (3). We report here that p26 HRF was abundantly expressed in murine peritoneal macrophages. Furthermore, the present study demonstrated that M-CSF acted as a potent stimulator for induction and secretion of this factor in macrophages in vitro and in vivo, suggesting a novel action of M-CSF on macrophage functions. The present study does not eliminate the possibility that other untested cytokines or factors may also up-regulate the p26 HRF production by macrophages. We paid special attention to the effect of GM-CSF, since this cytokine has been detected in bronchoalveolar (38) and nasal lavage (35) fluids of allergen-induced late phase reactions, where p26 HRF was also found (31, 36). However, GM-CSF did not increase the p26 HRF mRNA level, protein synthesis, and accumulation in macrophages (Fig. 5).

The p26 HRF and its human homolog are secretory proteins, while they do not have an obvious signal sequence at the NH\(_2\) termini (26, 27). The secretory mechanism is still unknown. The medium, collected after a 4-h cultivation of resident macrophages, contained a 30-kDa protein, which was immunoreactive to two Abs that recognize different epitopes of p26 HRF, and other immunoreactive protein bands were not observed. The p26 HRF has an N-glycosylation site (Asp-Xaa-Ser), corresponding to amino acids 51–53 (26, 27). This 30-kDa protein was likely to be an N-glycosylated p26, since OVA-elicited macrophages contained one or two 30-kDa proteins, which were immunoreactive to the two Abs against p26 HRF, and these proteins migrated to the same position as p26 HRF after treatment with N-glycosidase F (Fig. 8). However, when the 30-kDa protein, secreted into culture medium, was treated with N-glycosidase F, we could not obtain a consistent result, indicating that p26 HRF might undergo more complex modification besides N-glycosylation during secretory processes. Elucidation of the secretory processes of the HRF from macrophages is an important next step to understand the roles of the macrophage-derived HRF in allergic inflammation.

Next, we tested whether M-CSF actually up-regulated p26 HRF production by macrophages in vivo (Fig. 6). The p26 HRF has been suggested to participate in chronic allergic reactions (31, 36); therefore, we also examined the effects of M-CSF on macrophages of Ag-immunized mice. Repeated i.p. injections of M-CSF into normal mice could elicit macrophages expressing abundant p26 HRF, and higher amounts of p26 HRF were recovered from peritoneal lavage fluid of the mice, compared with those of control animals. Treatment of resident macrophages with LPS in vitro did not induce p26 HRF, while LPS-elicited peritoneal macrophages increased the intracellular level, as did M-CSF-elicited cells. The explanation for this may be that in vivo treatment with LPS may stimulate several target cells to produce multiple cytokines that can induce p26 HRF. In fact, LPS induces the synthesis and secretion of mouse M-CSF in vivo (46). This might also be the case in thioglycollate- and adjuvant-treated mice, since these treatments could not completely eliminate the effects of contaminated LPS and may directly or indirectly stimulate multiple target cells. However, the reactivity to M-CSF in peritoneal macrophages of OVA-immunized mice was strikingly different. A single injection of M-CSF markedly accelerated the p26 HRF induction, while it did not in the cells of LPS-, thioglycollate-, or adjuvant-treated mice, suggesting that there may be Ag-specific priming of macrophages for the enhanced production of p26 HRF by M-CSF. In fact, this accelerated response of OVA-elicited macrophages to M-CSF was clearly documented in vitro (Fig. 7): treatment of the OVA-elicited cells in culture with M-CSF accelerated the induction of p26 HRF and additionally enhanced the secretion of this factor. In this case, significant amounts of N-glycosylated p26 HRF were detected within the OVA-elicited cells 2 h after addition of M-CSF, while the posttranslationally modified forms were not observed in resident cells even after treatment with M-CSF. These results indicate that the immunization may prime macrophages for enhanced production of p26 HRF in response to M-CSF, although the molecular basis of this priming is not clear.

An i.p. challenge with OVA to OVA-sensitized mice markedly increased the p26 HRF level in the peritoneal cavity (Fig. 9). The time course of this elevation roughly coincided with the appearance of the late phase reaction. A similar increase could be produced by a single injection of M-CSF, and in this case, overproduction of p26 HRF was observed at even an earlier time point. Treatment of OVA-immunized mice with M-CSF also caused the accumulation of macrophages more rapidly than that with OVA (Fig. 10). It should be emphasized that challenge with M-CSF or rp26 HRF induced eosinophil migration, even in the absence of the Ag in sensitized mice, but not in normal animals. This phenomenon resembled a feature characteristic of the late phase allergic reaction. Furthermore, rp26 HRF injection initiated eosinophil recruitment more rapidly than with M-CSF, and the accumulation occurred without marked macrophage and lymphocyte infiltrations, suggesting that rp26 HRF may mediate allergic inflammations by facilitating eosinophil recruitment, which was recently reported to occur in human cells (36, 37). OVA challenge stimulates multiple pathways, including the production of IL-5 and eotoxin (30, 47); therefore, it promoted twofold severe eosinophilia, compared with that with M-CSF or rp26 HRF. In fact, mast cell accumulation was observed only by OVA challenge. Basophils appeared not to be involved in our murine model. It is unknown whether p26 HRF requires surface IgE\(^+\) on murine cells and which populations are target cells against this factor in mice. However, our results suggest that p26 HRF may be one of the important factors that trigger allergic inflammations, at least in part, by promoting eosinophil recruitment. At present, the exact nature for the interaction between IgE\(^-\) and p26 HRF, and the mechanism of action by p26 HRF, including a specific receptor for p26 HRF and intracellular signal events, remain unknown.

The Th2 profile of cytokines has been suggested to play a central role in the pathogenesis of chronic allergic inflammation, while the production of M-CSF in allergic subjects has not been well examined at the site of allergic disorders. In a mouse model of OVA-induced pulmonary eosinophilia, an OVA-challenge caused an early accumulation of macrophages in the lung (45). Human upper airway fibroblasts and epithelial cells release M-CSF, which enhances the survival of accumulated monocytes/macrophages in
the airway during inflammation (48). If the overproduction of p26 HRF by primed macrophages plays an important role in the development of late phase reaction, our results may call attention to a previously unrealized role of M-CSF in the pathogenesis of chronic allergic disorders.

References


