

Molecular Identification of IgE-Dependent Histamine-Releasing Factor as a B Cell Growth Factor¹

Hyung Sik Kang, Min Ju Lee, Hyunkeun Song, Seung Hyun Han, Yong Man Kim, Joo Young Im, and Inpyo Choi²

The culture supernatants of LK1 cells, murine erythroleukemia cells, showed B cell-stimulating activity. Purification and NH₂-terminal sequence analysis revealed that one of the candidates was murine IgE-dependent histamine-releasing factor (IgE-HRF), which is known to induce histamine from basophils. Recombinant IgE-HRF (rHRF) obtained from *Escherichia coli*- or 293-transformed embryonal kidney cells was tested for B cell-stimulating activity. Both rHRFs stimulated B cell proliferation in a dose-dependent manner. However, boiling or anti-HRF Ab abolished the B cell stimulatory effects of rHRF. Recombinant HRF showed strong synergistic effects with IL-2, IL-4, and IL-5 for B cell activation, with maximal activity in the presence of anti-CD40 Ab. Recombinant HRF increased MHC class II expression of B cells. It also increased Ig production from B cells. Treatment with polymyxin B, a neutralizing peptide antibiotic of LPS, did not reduce the activity of rHRF. In addition, FACS analysis using PE-conjugated rHRF showed that HRF bound to B cells. Recombinant HRF up-regulated the expression of IL-1 and IL-6 in B cells. In vivo administration of rHRF or the cDNA for rHRF increased total and Ag-specific Ig synthesis. Taken together, these results indicate that HRF stimulates B cell activation and function. *The Journal of Immunology*, 2001, 166: 6545–6554.

Activation of B cells during the different stages of immune responses is dependent on signals through the cell surface molecules, including Ag receptor, coreceptors, and cytokines such as IL-2, IL-4, and IL-5 in the case of murine B cells (1). In response to appropriate stimuli, B cells undergo at least three major steps, such as activation, growth, and differentiation.

Many different stimuli are known to induce B cell activation. LPS, viral hemagglutinins, CpG bacterial DNA, anti-Ig Ab, and PMA are well-known polyclonal B cell activators (2–5). However, their roles in B cell activation are not same. For example, anti-Ig Ab stimulates B1 cells to induce DNA synthesis (6). Maturation of blast cells into Ab-secreting cells requires T cell-derived differentiation factors. CD40 ligand (CD40L)³ expressed by T cells delivers important signals in B cells to regulate cell proliferation, production of Ig, Ig class switching, rescuing cells from apoptosis, and germinal center formation (7). The roles of CD40L in the regulation of the B cell response have been confirmed in CD40L-deficient mice (8, 9). However, CD40L-CD40 interaction does not account for all contact-dependent T cell help for B cells. CD40L-deficient T cells have been shown to induce the proliferation and differentiation of B cells successfully (10). CD30L (11) and BAFF (12) expressed by T cells induce B cell activation in a CD40-

independent manner. Recently, a B cell-specific transmembrane protein, RP-105, was also known to trigger B cell activation through a unique pathway that was different from IgM-mediated or CD40-mediated pathways (13).

Histamine-releasing factors (HRFs) are a group of factors that release histamine and other mediators from mast cells and basophils. It has been reported that HRF is involved in the pathogenesis of allergic diseases. There are two types of HRFs: one induces histamine release in the presence of IgE, and the other operates independently of IgE. IgE-dependent HRF (IgE-HRF) was first molecularly identified by MacDonald et al. (14). This HRF is a unique molecule with no homology to any known IL, chemokine, or Ag. Subsequent studies have been shown that IgE-HRF plays an important role in perpetuating late phase allergic reaction (15, 16). In addition, IgE-HRF stimulates the production of IL-4 and IL-13 from all basophils in the presence of IgE (17, 18). However, additional data suggest that IgE-HRF has a unique signaling pathway and binds to a specific receptor other than IgE (19). The molecular information for the IgE-HRF receptor is not known yet.

Recent results (11–13) of CD40- or T cell-independent B cell stimulatory factors impelled us to search for novel B cell stimulatory factors. In this regard we recently established a cell line, LK1 (20), derived from the spleen of a mouse that showed typical splenectasia and expressed several cytokines. In addition, the culture supernatants of LK1 cells stimulated B cell proliferation compared with those of similar lineage cell lines. As an effort to purify novel B cell stimulatory factors secreted from this cell line, we identified IgE-HRF as a B cell activation factor. IgE-HRF stimulated B cell growth and differentiation, which was different from the effects of LPS. It bound to B cells and induced cytokine production from B cells.

Materials and Methods

Reagents and Abs

LPS, MTT, and polymyxin B sulfate (PMB) were purchased from Sigma (St. Louis, MO). Recombinant murine IL-2, IL-4, and IL-5 were purchased from R&D Systems (Minneapolis, MN). Anti-mouse CD40, anti-mouse

Laboratory of Immunology, Korea Research Institute of Bioscience and Biotechnology, Taejeon, Republic of Korea

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² Address correspondence and reprint requests to Dr. Inpyo Choi, Laboratory of Immunology, Korea Research Institute of Bioscience and Biotechnology, Eoun-Dong 52, Yusong, Taejeon 305-333, Republic of Korea. E-mail address: ipchoi@kribb4680.kribb.re.kr

³ Abbreviations used in this paper: CD40L, CD40 ligand; HRF, histamine-releasing factor; SA/PE, streptavidin-conjugated PE; IgE-HRF, IgE-dependent HRF; PMB, polymyxin B sulfate; PVDF, polyvinylidene fluoride; MBP, maltose-binding protein; LAL, *Limulus* amoebocyte lysate; rHRF, recombinant IgE-HRF.

IgM, and anti-mouse class II Ab were purchased from Immunotech (Miami, FL). Sephadex G-10 and Mono-Q column were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). The mAb against Thy 1.2 was obtained from the ascites of HO-13-4 hybridoma. Rabbit complement was purchased from Serotec (Oxford, U.K.). FITC-conjugated anti-CD3 and anti-CD45R/B220 were purchased from PharMingen (San Diego, CA). For the production of anti-HRF polyclonal Ab, purified recombinant IgE-HRF (rHRF; 250 μ g) was mixed with the same volume of IFA (Sigma) and injected i.m. three times into a male rabbit (New Zealand White) at 2-wk intervals. The activity and specificity of this Ab were assessed by ELISA and Western blotting. PV200 malaria Ag peptide was a gift from David C. Kaslow, National Institutes of Health (Bethesda, MD).

Cell culture and DNA transfection

LK1 cells were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FBS (Life Technologies), penicillin (100 U/ml), streptomycin (100 μ g/ml), and L-glutamine (0.3 mg/ml). No additional growth factors were used, and cells were fed twice weekly by partial replacement with fresh medium. For transient transfection assays, HRF cDNA was subcloned into the mammalian expression vector pFLAG-CMV, and 293 cells were transfected with the plasmids by the calcium phosphate-DNA coprecipitation method (21).

Purification of HRF

Culture supernatants were collected from 25 liters of LK1 cells (1×10^6 cells/ml) cultured in serum-free and protein-free hybridoma medium (Sigma) for 24 h. The culture supernatants were concentrated by ultrafiltration (YM-10 membrane, Amicon, Beverly, MA) to 10 ml and dialyzed for 24 h in 10 mM Tris-HCl (pH 7.6). The dialyzed sample was then applied at a flow rate of 60 ml/h to a Mono-Q HR5/5 column equilibrated in 10 mM Tris-HCl (pH 7.6), and the adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 1 M. Aliquots of each fraction were assayed for B cell-stimulating activity. The partially purified proteins possessing biological activity were collected and further purified onto a Mono-Q column with a modification of a linear gradient of NaCl. The fractions showing maximal biological activity were concentrated by 10% TCA (Sigma) and applied to the SDS-PAGE. Then proteins were electrophoretically transferred from the gel to polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The amino acid sequences of NH₂-terminal region were determined by automated Edman degradation using a gas phase sequencer.

Expression and purification of rHRF in Escherichia coli

The HRF-encoding region was obtained from the human keratinocyte cDNA library using PCR and cloned into the pMAL-c2 expression vector (New England Biolabs, Beverly, MA). PCR primers were made to introduce an *Eco*RI site at the initiation codon (5'-cggaattc ATG ATC ATC TAC CGG GAC) and a *Sall* site downstream of the stop codon (5'-cgcgtc gac TTA ACA TTT CTC CAT CTC TAA GCC). The thrombin cleavage site (gaattc CTG GTT CCG CGT GGA TCC gaattc) was inserted into the *Eco*RI site of pMAL-c2-HRF for the cleavage of maltose-binding protein (MBP)-HRF fusion protein (pMALc2-Thr-HRF). *E. coli* strain JM109, containing the pMALc2-Thr-HRF was grown in Luria Bertoni broth supplemented with 0.2% glucose. When the OD at 600 nm was 0.8, isopropylthio- β -D-galactoside was added to a final concentration of 1 mM and incubated for 24 h at 20°C. The cells were centrifuged and disrupted by sonication in 20 mM Tris (pH 7.5). The supernatants were filtered with a 0.45- μ m pore size filter, and the MBP-HRF fusion protein was isolated using the amylose column (New England Biolabs). The fusion protein was cleaved by thrombin treatment and then isolated into MBP and HRF using a DEAE-Sepharose column (Pharmacia Biotech). Endotoxin was removed using a PMB-agarose column (Sigma) according to the method suggested by the manufacturer.

Limulus amoebocyte lysate (LAL) assay

Detection of endotoxin was determined by LAL assay. The assay kit was purchased from Hemachem (Ringmer, East Sussex, U.K.), and the amount of endotoxin in rHRF or MBP was measured using LAL reagent according to the manufacturer's instruction. Control standard endotoxin was used as a positive control, and pyrogen-free 10 mM Tris (pH 8.0) as a negative control.

Isolation of splenic B cells

Splenic B cells were isolated from 6- to 8-wk-old BALB/c mice as previously described (22). RBC were removed by treatment of RBC lysis buffer (Sigma). Splenic T cells were depleted by treatment of anti-Thy 1.2 and

rabbit complement-mediated lysis (Serotec). The cells were applied to a Sephadex G-10 column to remove cell lysates and other cell populations except B cells (Pharmacia, Piscataway, NJ). The purity of B cells (B220-positive population) was ~90%.

Cell proliferation assay

Isolated splenic B cells (2×10^5 /well) were plated on 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) in 100 μ l of RPMI 1640 supplemented with 10% FBS, and 100 μ l of test samples were added. To destroy the biological activity of HRF, splenic B cell culture supernatants were treated with 1 mg/ml trypsin for 1 h at 25°C or boiled for 10 min. For the [³H]thymidine uptake assay, cells were incubated for 72 h in a humidified 5% CO₂ incubator at 37°C. The cells were pulsed with 0.5 μ Ci of [³H]thymidine (sp. act., 84.8 Ci/mmol; New England Nuclear, Boston, MA) for the last 6 h of incubation and were harvested onto a glass-fiber filter using an automated cell harvester (Inotech, Zurich, Switzerland). The amount of radioactivity incorporated into the DNA was determined using a liquid scintillation counter (LS 6000A; Beckman, Palo Alto, CA). For the MTT assay, 100 μ g of MTT was added to the each well, and the MTT assay was performed as previously described (23).

Ig production from splenic B cells

Splenic B cells were incubated with 1 μ g/ml of LPS or 500 ng/ml of HRF in the presence or the absence of PMB (1 μ g/ml) for 3 days. After incubation, cell culture supernatants were collected, and Ig production was measured with an mAb-based mouse Ig isotyping kit (PharMingen) according to the manufacturer's instruction.

Flow cytometric analysis

Splenic B cells treated as indicated were washed with staining buffer (PBS containing 3% FBS and 0.1% Na₂S₂O₃) and stained with FITC-conjugated anti-CD3, anti-B220, or anti-MHC class II Ab for 30 min on ice. After incubation, the cells were washed and analyzed using a flow cytometer (Becton Dickinson, Mountain View, CA).

HRF binding assay

Whole splenocytes or splenic B cells (1×10^6 cells) were incubated with 1 μ g/ml of LPS for 24 h at 37°C. After incubation, the cells were washed and incubated with biotin-conjugated rHRF for 30 min on ice. For competition experiments, a 10-fold excess of unconjugated rHRF or 2 μ g/ml of anti-mouse IgE Ab was added to the cells together with biotin-labeled rHRF. Cells were washed with staining buffer, and streptavidin-conjugated PE (SA/PE) was added to each sample, followed by incubation for 30 min on ice. The cells were washed and incubated with FITC-labeled anti-B220 or anti-CD3 Ab for 30 min on ice. Binding of HRF was analyzed by flow cytometry.

Western blot analysis

Western blotting was conducted as previously described (24). 293 cells transiently transfected with pCMV-flag-human HRF were lysed in lysis buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 1 mM Na₃VO₄, 1 mM PMSF, 100 μ g/ml aprotinin, and 1 μ g/ml leupeptin). The protein concentrations were determined using Bradford reagent (Bio-Rad). Cell lysates containing equal amounts of protein were resolved by 10% PAGE and transferred to an immunoblot PVDF membrane (Bio-Rad). The blot was treated with anti-mouse HRF Ab followed by incubation with peroxidase-conjugated secondary Ab. The Ag-Ab complexes were detected using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ). Following electroblotting, the blot was stained with Coomassie blue to normalize the protein concentrations of each lane.

RT-PCR analysis

Total cellular RNA was extracted by using RNeasy B (Tel-Test, Friendswood, TX) according to the manufacturer's instruction. Aliquots (3 μ g) of total RNA were transcribed into cDNA at 37°C for 1 h in a total volume of 20 μ l with 2.5 U of Moloney murine leukemia virus reverse transcriptase (Roche, Mannheim, Germany). Reverse transcribed cDNA samples were added to a PCR mixture consisting of Takara 10 $\times$ PCR buffer, 0.2 mM dNTP, 0.5 U of Taq DNA polymerase, and 10 pmol of primers of each cytokines. The primer sequences are as follows: IL-1, 5'-GAAGGGCTGCTTCCAAACCTTTGACC-3' and 5'-TGTGGATTGAGGTGGATTCTTGC-3'; IL-2, 5'-AACAGCGCAC CCACTTCAA-3' and 5'-TTGAGATGATGCTTTGACA-3'; IL-4, 5'-GTCT GCTGTGGCATATTCTG-3' and 5'-GGCATTTCATTTCAGATTC-3'; IL-6, 5'-ATGAAGTCTCTCTGCAAGA-3' and 5'-GGTTTGGCCGAGTA

CATCTCAA-3'; IL-10, 5'-TCCTTAATGCAGGACTTTAAGGGT TACTTG-3' and 5'-GACACCTTGGTCTGGAGCTTATTAATC-3'; TNF- α , 5'-GGCAGGTCTACTTGGAGTCATTGC-3' and 5'-ACATTC GAGGCTCCAGTGAATTCGG-3'; TGF β 1, 5'-TGGACCGCAACAACGC CATCTATGAGAAAACC-3' and 5'-TGGAGCTGAAGCAATAGTTGGTA TCCAGGGCT-3'; and β -actin, 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCC TTAATGTCACGCACGATTTC-3'. All PCR mixtures were heated to 95°C for 1 min and cycled 30 times at 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min, followed by an additional extension step at 72°C for 10 min. PCR products were electrophoresed and visualized by ethidium bromide staining.

EMSA

Splenic B cells (1×10^7) were stimulated with 1 μ g/ml of LPS or 500 ng/ml of rHRF in the presence or the absence of anti-CD40 Ab (250 ng/ml) for 6 h. The nuclear extracts were prepared according to the procedure previously described (25). DNA mobility shift assays were performed using double-stranded oligonucleotides comprising the consensus sequences for NF- κ B (5'-GGGAGTTGAGGGGACTTCCAGGC-3'). Oligonucleotides were terminal-labeled with [α - ^{32} P]dCTP using a Klenow fragment of DNA polymerase I. Aliquots of nuclear extracts (5 μ g) were incubated at room temperature for 30 min with labeled oligonucleotides in a total volume of 20 μ l under following conditions: 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 2 μ g of poly(dI:dC). DNA-protein complexes were electrophoresed on a 6% polyacrylamide gel, and the gel was dried and autoradiographed.

In vivo treatment of HRF protein or plasmid DNA

To investigate in vivo effects of rHRF and the cDNA for HRF on Ig production, splenocyte proliferation, and cytokine expression, BALB/c female mice (three mice per group) between 6 and 8 wk of age were injected i.m. with 20 μ g of rHRF or with 50 μ g of pcDNA-HRF in combination with 20 μ g of PV200 peptide Ag on days 21, 31, and 41. The mice were sacrificed on day 10 after the last injection, and antisera were collected for determination of Ig production. Anti-PV200 Ab production was determined by ELISA. Splenocytes derived from these mice were removed and then stimulated with 500 ng/ml of rHRF plus 250 ng/ml of anti-CD40 Ab for 3 days.

The cells were pulsed with 0.5 μ Ci of [^{3} H]thymidine for the last 6 h of incubation, and cell proliferation was determined by the amount of radioactivity incorporated into the DNA.

Statistical analysis

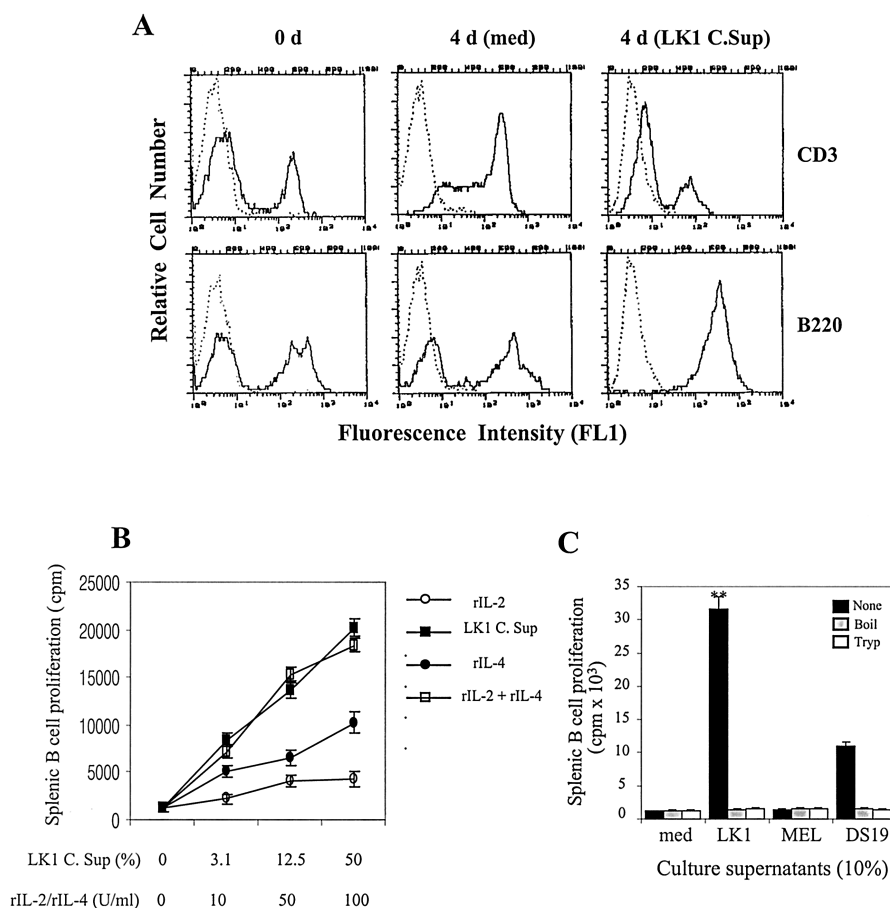
For statistical analysis of data, *p* values were analyzed using the paired Student's *t* test program (StatView 5.1; Abacus Concepts, Berkeley, CA). Results were considered statistically significant when *p* < 0.05.

Results

Identification of IgE-HRF as a B cell stimulatory factor in the culture supernatants of LK1 cells

Previously we established a murine erythroleukemia cell line, LK1 cells (20). Based on RT-PCR, LK1 cells produce cytokines such as IL-5, IFN- γ , and TNF- α (20). When the culture supernatants of LK1 cells were incubated with whole spleen cells for 4 days, B cells multiplied, but the T cell population decreased, suggesting that LK1 cells secrete B cell stimulatory factors that decrease the proportional T cell population or that there are some inhibitory factors for T cell proliferation in their culture supernatants (Fig. 1A). They also increased the proliferation of purified B cells dose-dependently (Fig. 1B). Combined treatment of rIL-2 and rIL-4 increased B cell proliferation in a dose-dependent manner also. The B cell-stimulating activity was abolished by boiling and trypsin treatment of the culture supernatants (Fig. 1C). The culture supernatant or other established murine erythroleukemia cell lines, such as MEL and DS19, showed much less B cell proliferation activity (Fig. 1C). As an effort to isolate potential B cell-stimulating factors, 25 l of these supernatants were concentrated, and the proteins

FIGURE 1. Effects of culture supernatants of LK1 cells on B cell proliferation. **A**, Whole splenocytes were cultured in the presence (LK1 C. Sup) or the absence (med) of LK1 cell culture supernatants for 4 days and stained with FITC-conjugated anti-CD3 or anti-B220 Ab as described in *Materials and Methods*. The cells were analyzed by flow cytometry. **B**, Splenic B cells (2×10^5 /well) were plated on 96-well plates in 100 μ l of RPMI 1640 supplemented with 10% FBS, and 100 μ l of diluted LK1 cell culture supernatants or cytokines were added. After 3-day incubation, cell proliferation was measured by MTT assay. **C**, Splenic B cells were incubated with boiled or trypsinized culture supernatants of mouse erythroleukemia cell lines (LK1, MEL, DS19) for 3 days. The cells were pulsed with 0.5 μ Ci of [^{3} H]thymidine for the last 6 h of incubation time, and cell proliferation was determined by the amount of radioactivity incorporated into the DNA. Results are representative of five independent experiments and are expressed as the mean $\pm$ SD of duplicate cultures. **, *p* < 0.01 vs all other groups.



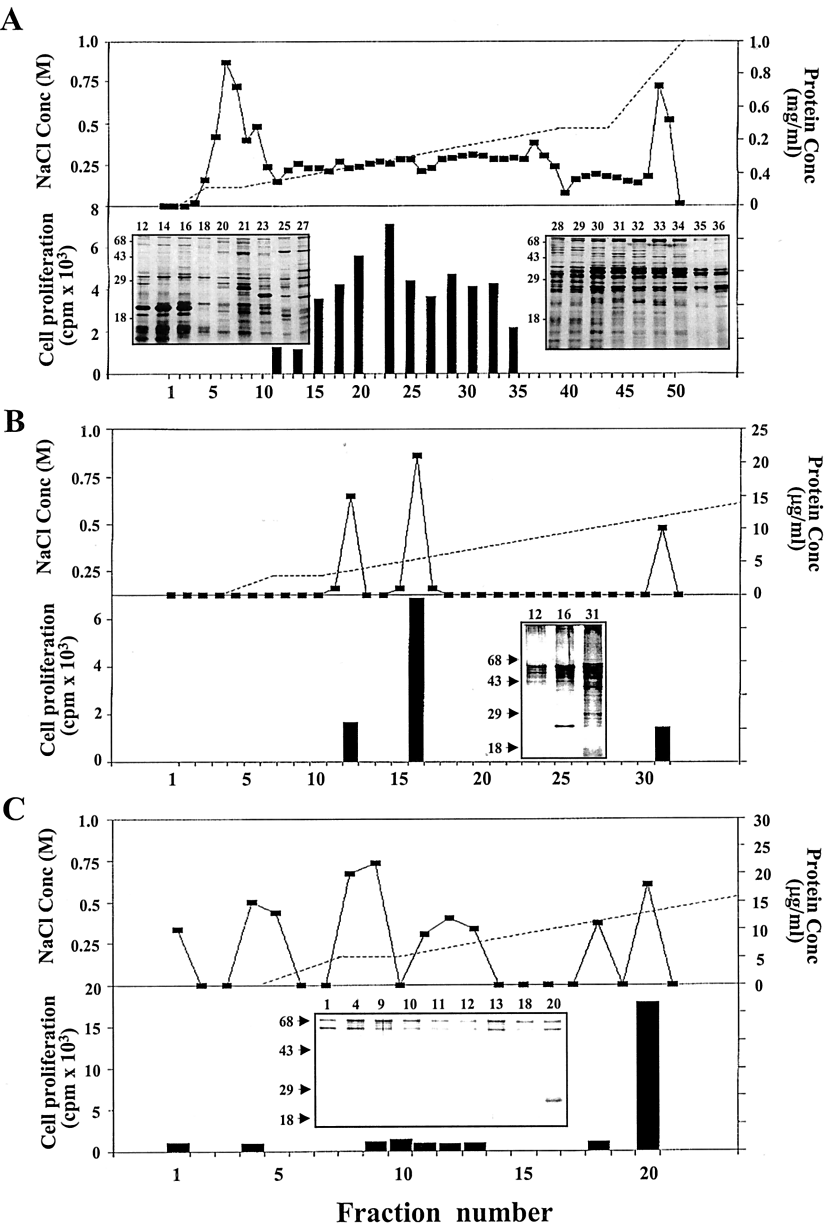
contained therein were fractionated by Sephadex G-75 gel filtration and Mono-Q anion exchange chromatography (Fig. 2). Fractions 16–35 (Fig. 2A) were pooled and then rechromatographed. Fraction 16 (Fig. 2B) possessing biological activity was further purified. Finally, fraction 20 (Fig. 2C) showing B cell-proliferating activity was subjected to SDS-PAGE and blotted onto a PVDF membrane for peptide sequencing. The NH₂-terminal amino acids (NH₂-MIIYRDLISHD-COOH) revealed 100% homology to those of murine IgE-HRF. IgE-HRF is known to be involved in allergic reaction and to stimulate the production of IL-4 and IL-13 from basophils, but nothing has been known about its effects on B cell function.

Effects of HRF on the proliferation and Ig production of B cells

To prove its biological activity further, the cDNA for murine HRF was subcloned into the pMAL-c2 plasmid, expressed as a fusion protein with MBP, and isolated from MBP by thrombin treatment and column chromatography (Fig. 3A). To remove the endotoxin in the rHRF preparation, PMB-agarose column chromatography was performed, and the endotoxin level was measured by LAL

assay. Endotoxin activity in the sample was removed to a level below the limit of detection (<1 pg/μg). Recombinant HRF stimulated mouse splenic B cell proliferation dose-dependently, with maximal activity at 100 μg/ml (Fig. 3B). Control MBP had minimal effects at high concentrations. This activity was completely abolished by boiling and anti-HRF Ab treatment (Fig. 3C). HRF was also transiently expressed in a mammalian cell line, 293 transformed embryonal kidney cells (Fig. 3D). Culture supernatants of HRF-transfected cells showed B cell stimulatory effects in a dose-dependent manner, but normal medium or culture supernatants of vector-transfectants had minimal effects (Fig. 3E). However, murine HRF had no stimulatory effect on human B lymphocytes (data not shown). IL-2, IL-4, IL-5, anti-CD40 Ab, and anti-IgM Ab are well-known stimulators of murine B cells. Next, the combination effects of rHRF on B cell proliferation were assayed in the presence of these B cell stimulators. In the presence of anti-CD40 Ab (250 ng/ml), rHRF increased B cell proliferation synergistically, and its activity peaked at 500 ng/ml (Fig. 4A). Thereafter, rHRF at a concentration of 500 ng/ml was used in the cotreatment experiments with anti-CD40 Ab and other stimulators. Recombinant

FIGURE 2. Purification of HRF from the culture supernatants of LK1 cells. *A*, LK1 cell culture supernatants were concentrated by ultrafiltration and dialyzed for 24 h as described in *Materials and Methods*. The dialyzed sample was then applied to a Mono-Q column, and the adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 1 M. Aliquots of each fraction were assayed for splenic B cell proliferation and protein determination. The fractions showing biological activity were subjected to SDS-PAGE and visualized by silver staining. *B*, Fraction 16 was further purified by the modification of linear NaCl gradient. *C*, Fraction 20 was transferred from the SDS-PAGE gel to a PVDF membrane for amino acid sequencing.



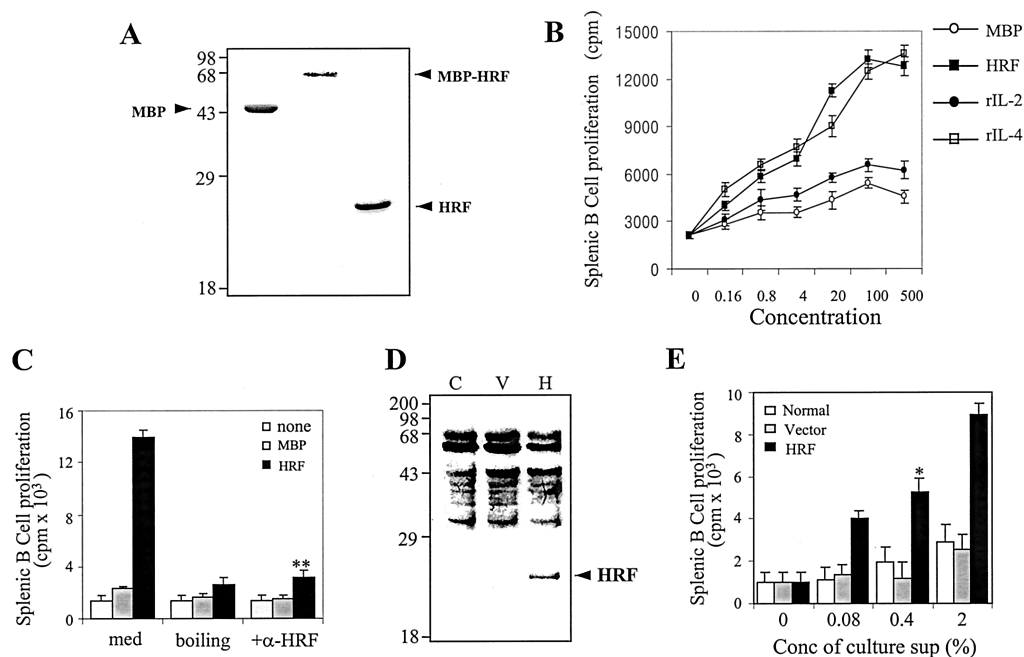


FIGURE 3. B cell stimulatory activity of rHRF. *A*, The MBP-HRF fusion protein was isolated by the amylose column and cleaved by thrombin treatment. It was further separated using DEAE-Sepharose column and 10% SDS-PAGE. *B*, Splenic B cells were incubated with HRF ($\mu\text{g/ml}$), MBP ($\mu\text{g/ml}$), or cytokines (U/ml) at various concentrations for 3 days. Cell proliferation was determined by [^3H]thymidine incorporation. *C*, Splenic B cells were treated with 100 $\mu\text{g/ml}$ of HRF or MBP after boiling or anti-HRF-Ab (1/500 dilution) pretreatment. After 3 days of incubation, cell proliferation was determined as described. **, $p < 0.01$ vs rHRF treatment (med). *D*, Transient HRF-transfected 293 cells were lysed in lysis buffer. Cell lysates were subjected to SDS-PAGE and transferred electrophoretically to a PVDF membrane. The membrane was screened with anti-HRF Ab followed by visualization with enhanced chemiluminescence. C, Normal control; V, vector transfectants; H, HRF transfectants. *E*, Splenic B cells were treated with the culture supernatants of HRF- or vector-transfected 293 cells at various concentrations for 3 days. Data are representatives of five independent experiments and represent the mean $\pm$ SD of duplicate cultures. *, $p < 0.05$ vs 0.08% of HRF-transfected 293 cells.

HRF showed mild synergistic effects with IL-2, IL-4, and IL-5 (Fig. 4*B*). Maximal activity was observed in the combination of cytokines, rHRF, and anti-CD40 Ab. Homotypic aggregation of B cells was examined by microscopic observation (Fig. 4*C*). Weak aggregation was observed when B cells were cultured with the

culture supernatant of LK1 cells (Fig. 4*C*, *b*) or MBP-HRF (Fig. 4*C*, *d*). The maximal homotypic aggregation of B cells was observed when B cells were cultured in the presence of MBP-HRF, anti-CD40, and anti-IgM Ab (Fig. 4*C*, *f*), even though MBP plus anti-CD40 and anti-IgM Ab had minimal effects (Fig. 4*C*, *e*). MHC

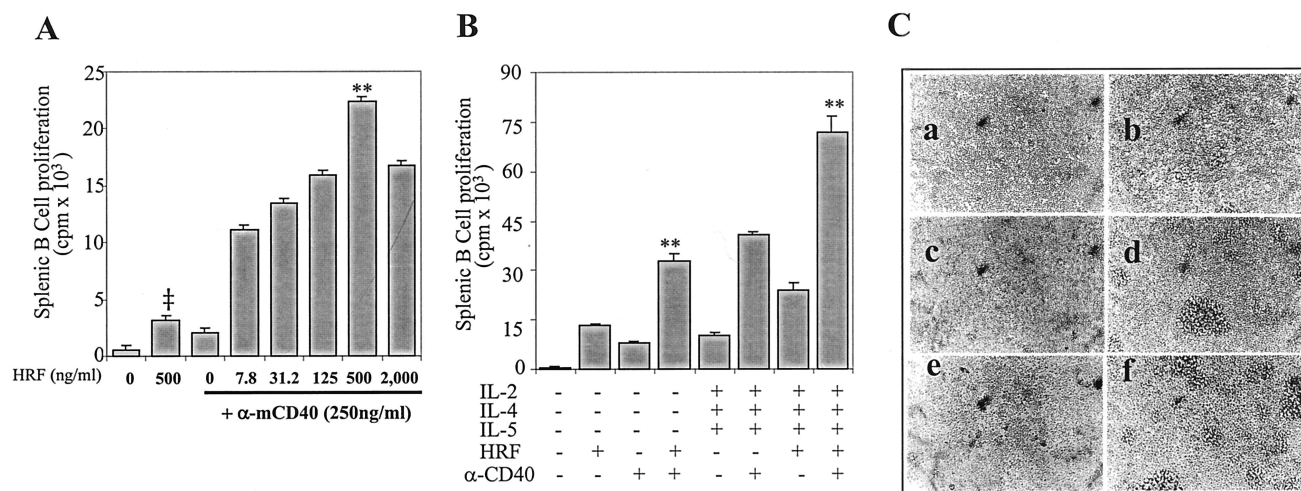


FIGURE 4. The combined effects of rHRF with other B cell activators on B cell proliferation. *A*, Splenic B cells were treated with rHRF at various concentrations in the presence or the absence of anti-mouse CD40 Ab (250 ng/ml). After 3 days of incubation, cell proliferation was determined by [^3H]thymidine incorporation. Data are representative of three independent experiments and are the mean $\pm$ SD of duplicate cultures. †, $p < 0.05$ vs no rHRF group; **, $p < 0.01$ vs all other groups. *B*, The cells were stimulated with various cytokines (100 U/ml) or rHRF (500 ng/ml) in the presence or the absence of anti-CD40 Ab (250 ng/ml) for 3 days. Cell proliferation was determined as described. **, $p < 0.01$ vs all other groups. *C*, After 3 days of incubation, homotypic aggregation of splenic B cells was observed by light microscopy ($\times 100$). *a*, Medium; *b*, LK1 cell culture supernatants (10%); *c*, MBP (100 $\mu\text{g/ml}$); *d*, MBP-HRF (100 $\mu\text{g/ml}$); *e*, MBP (100 $\mu\text{g/ml}$), anti-CD40 (250 ng/ml), and anti-IgM (100 ng/ml); *f*, MBP-HRF (100 $\mu\text{g/ml}$), anti-CD40 (250 ng/ml), and anti-IgM (100 ng/ml).

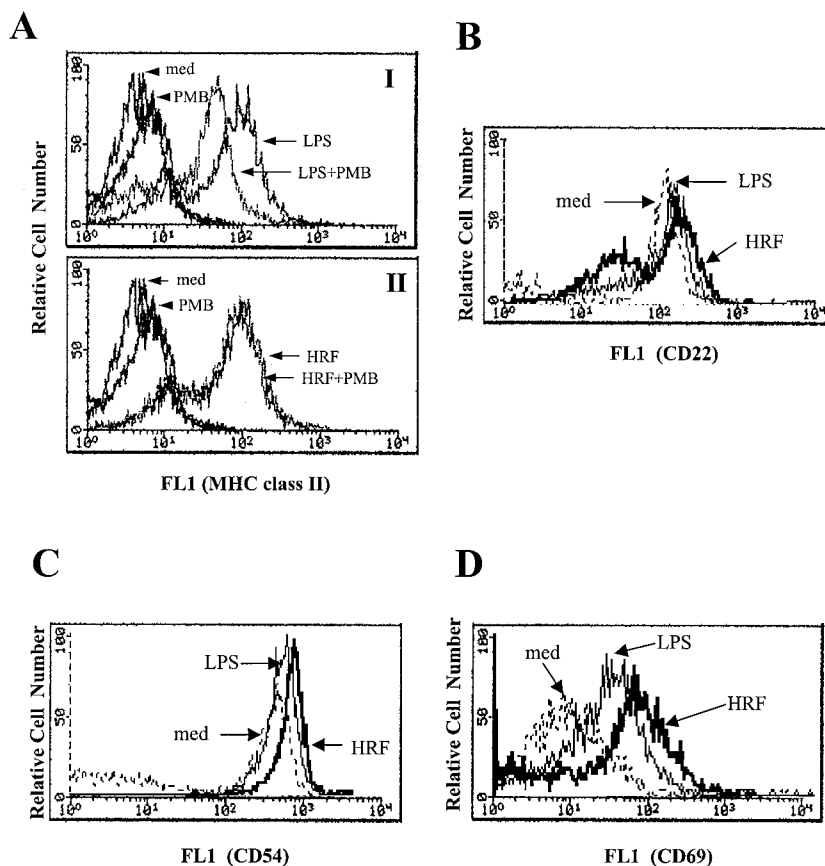


FIGURE 5. Effects of rHRF on the expression of B cell surface molecules. A, Splenic B cells were cultured with 1 μ g/ml of LPS (I) or 500 ng/ml of rHRF (II) in the presence or the absence of 1 μ g/ml PMB for 3 days. The cells were stained with FITC-conjugated anti-MHC class II Ab as described in *Materials and Methods*. MHC class II expression was analyzed by flow cytometry. The expression of other surface molecules, such as CD22 (B), CD54 (C), and CD69 (D), was analyzed as described above. One of four similar experiments is shown.

class II expression is another marker for B cell activation (13). When B cells were treated with LPS or rHRF, MHC class II expression was elevated (Fig. 5A). The effects of LPS on MHC class II expression were abolished by PMB (Fig. 5AI), a cyclic cationic peptide antibiotic that neutralizes the biological activity of LPS (26, 27), but those of rHRF were not affected by PMB (Fig. 5AII), suggesting that the effects of rHRF are different from those of LPS. Recombinant HRF also moderately increased the expression of other B cell surface molecules, such as CD22 (Fig. 5B) and CD54 (Fig. 5C), which are related to cell adhesion. Interestingly, rHRF significantly induced CD69 expression, which is the earliest leukocyte activation Ag and is expressed on activated B cells (28) (Fig. 5D), but it had little effect on CD23 and CD44 expression (data not shown). Next, Ig production from B cells was monitored by isotype-specific ELISA. Recombinant HRF induced Ig production from B cells (Table I). In comparison with LPS, rHRF induced less IgG, or IgA, but it induced more IgM in the absence or the

presence of anti-CD40 Ab, suggesting that rHRF induces a different stage of B cell differentiation compared with LPS. PMB abolished the effects of LPS on Ig production dramatically, except for IgM, but it had no effect on those of rHRF. HRF enhanced IL-4 and IL-13 secretion by human basophils (18), and it was observed that HRF induced IL-6 and IL-10 gene expression in B cells (see below). Its effects on Ig production may be due to the induced cytokines. When anti-IL-6 Ab or anti-IL-10 Ab was added to the culture, rHRF-induced IgM production was reduced partially ($19.5 \pm 5.3\%$ reduction by anti-IL-6 Ab and $14.4 \pm 2.7\%$ reduction by anti-IL-10 Ab, respectively).

Binding of HRF on B cells

Based on its effects on B cell activation, it is likely that the HRF-binding molecule would be expressed on B cells. Binding of HRF was monitored using SA/PE-conjugated rHRF (HRF-SA/PE) and anti-B220 Ab. HRF-SA/PE bound B cells as expected compared

Table I. HRF-induced Ig production from splenic B cells^a

Ig Class	Med	LPS	HRF	PMB			Anti-CD40		
				–	+LPS	+HRF	–	+LPS	+HRF
IgG1	9.4 $\pm$ 2.1	27.0 $\pm$ 2.6	25.4 $\pm$ 1.8	9.4 $\pm$ 2.1	21.0 $\pm$ 1.8	25.4 $\pm$ 1.5	15.2 $\pm$ 2.4	43.1 $\pm$ 1.1	38.9 $\pm$ 1.3
IgG2a	8.0 $\pm$ 1.3	27.2 $\pm$ 2.8	16.7 $\pm$ 2.2	7.7 $\pm$ 1.3	13.9 $\pm$ 2.8	18.8 $\pm$ 2.0	11.5 $\pm$ 1.1	31.2 $\pm$ 1.8	27.4 $\pm$ 2.4
IgG2b	9.8 $\pm$ 2.5	39.8 $\pm$ 1.9	23.4 $\pm$ 1.7	9.6 $\pm$ 1.7	21.0 $\pm$ 1.4	20.7 $\pm$ 2.3	12.3 $\pm$ 1.2	45.6 $\pm$ 2.6	34.1 $\pm$ 2.2
IgG3	19.9 $\pm$ 1.8	65.9 $\pm$ 2.0	61.8 $\pm$ 1.6	17.6 $\pm$ 2.2	41.8 $\pm$ 1.1	56.2 $\pm$ 2.1	38.1 $\pm$ 2.5	69.3 $\pm$ 3.1	70.2 $\pm$ 2.6
IgM	194.0 $\pm$ 8.3	222.0 $\pm$ 6.2	242.4 $\pm$ 9.4	176.5 $\pm$ 8.1	226.0 $\pm$ 4.3	214.0 $\pm$ 5.1	215.2 $\pm$ 5.4	251.6 $\pm$ 8.8	307.7 $\pm$ 9.1
IgA	12.9 $\pm$ 1.9	58.4 $\pm$ 1.9	39.5 $\pm$ 1.6	12.7 $\pm$ 1.9	31.7 $\pm$ 1.6	39.9 $\pm$ 1.7	34.0 $\pm$ 2.7	59.3 $\pm$ 2.4	46.8 $\pm$ 1.2
IgE	0.1 $\pm$ 0.2	1.6 $\pm$ 0.4	1.8 $\pm$ 0.3	0.1 $\pm$ 0.4	0.8 $\pm$ 0.3	1.9 $\pm$ 0.5	0.3 $\pm$ 0.1	3.2 $\pm$ 0.8	4.1 $\pm$ 0.5

^a Splenic B cells (5×10^5) were incubated with 1 μ g/ml LPS or 500 ng/ml HRF in the presence or absence of 1 μ g/ml PMB for 3 days. After incubation, cell culture supernatants were collected and measured by ELISA as described in *Materials and Methods*. Results (ng/ml) are expressed as mean $\pm$ SD of triplicate determinations. One of three similar experiments is shown.

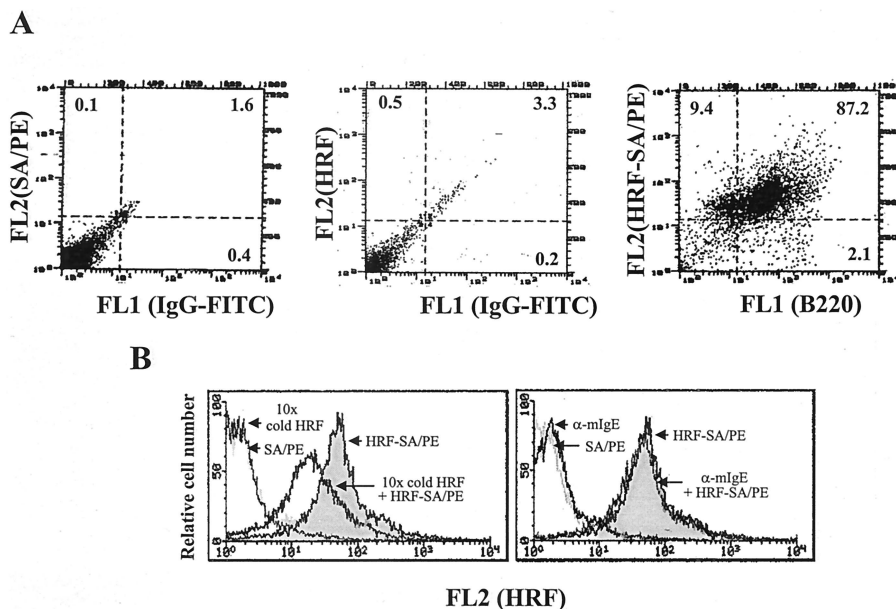


FIGURE 6. Binding of rHRF on B cells. *A*, Splenic B cells were incubated in the presence of biotin-conjugated rHRF for 30 min on ice. Cells were washed, and SA/PE was added. Following incubation for 30 min on ice, cells were washed and incubated with FITC-labeled anti-B220 Ab for 30 min on ice. *B*, For competition experiments, a 10-fold excess of unconjugated rHRF (left, 10 $\times$ cold HRF) or anti-mouse IgE Ab (right) was added to the cells together with biotin-labeled rHRF and SA-PE. Binding of HRF was analyzed by flow cytometry.

with IgG plus SA/PE or unlabeled HRF control (Fig. 6A). Its binding was competed with unlabeled cold rHRF (Fig. 6B). Previously, HRF was thought to bind and stimulate target cell by interacting with IgE molecules on the surface of these cells. However, additional studies showed that HRF stimulates cells by binding to an unknown receptor, which is distinct from IgE. When cells were incubated with anti-IgE Ab, there was no difference in HRF binding to B cells, further suggesting that HRF binds to cells through its own receptor, which is not related to IgE.

Effects of HRF on gene expression of B cells

Murine B cells produce several cytokines in response to mitogens. Recombinant HRF induced IL-1, IL-6, and IL-10 expression from B cells in the absence or the presence of anti-CD40 Ab, but it had no effect on IL-2, TNF- α , and TGF- β expression (Fig. 7A). NF- κ B is known to be one of important transcription factors regulated during B cell activation. NF- κ B was activated slightly by

rHRF alone, but it was dramatically activated by rHRF in combination with anti-CD40 Ab (Fig. 7B).

In vivo stimulatory effects of HRF on Ig and cytokine production

To analyze the in vivo effects of HRF on Ig production and cytokine expression, BALB/c mice were injected with pcDNA-HRF or rHRF in combination with PV200 peptide Ag. Antisera from these mice were collected for determination of Ig production. As shown in Table II, total Ig production was increased by rHRF treatment. Consistent with in vitro results, IgM production was dominant. When pcDNA-HRF was injected, a similar pattern was observed compared with pcDNA or pcDNA-IL-18 injection. Ag-specific Ig production was monitored by PV200 ELISA (Fig. 8A). As expected, rHRF increased the PV200-specific Ig production. However, infusion of IgE with rHRF had no effect on Ig production in vivo (data not shown). Next, splenocytes from immunized mice were restimulated with anti-CD40 Ab in vitro. As shown in Fig.

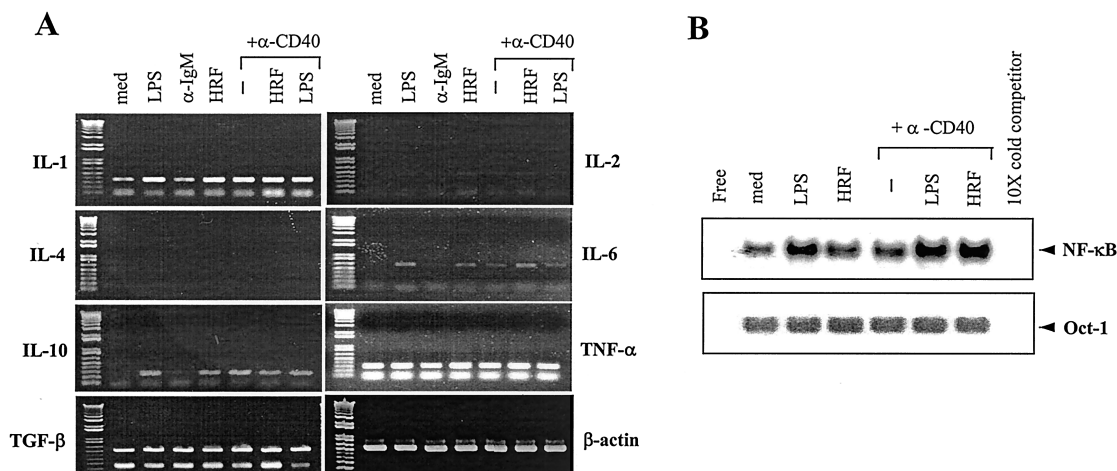


FIGURE 7. Effects of rHRF on cytokine expression and NF- κ B activation in B cells. *A*, Splenic B cells were treated with 1 μ g/ml of LPS, 100 ng/ml of anti-IgM, or 500 ng/ml of rHRF in the presence or the absence of anti-CD40 Ab (250 ng/ml) for 6 h. Total cytoplasmic RNA was isolated from the cells, and RT-PCR using cytokine-specific primers was performed as described in *Materials and Methods*. *B*, Splenic B cells were treated as described in *A*. Nucleic extracts were isolated from the cells, and 10 μ g of them were incubated for 30 min with ^{32} P-labeled NF- κ B probe. EMSA was performed as described in *Materials and Methods*. Equal loading of nuclear extracts was assessed by constitutively expressed Oct-1 binding. Free, Free probe; 10 $\times$ cold competitor, competition analysis with a 10-fold excess of unlabeled NF- κ B probe. The experiments were repeated three times with similar results.

Table II. *In vivo* effects of rHRF or the cDNA for HRF on Ig production^a

Ig Class	Normal	PV200	PV200 + rHRF	rHRF	pcDNA	pcDNA - HRF	pcDNA - IL-18
IgG1	23.6 ± 3.4	31.9 ± 1.8	45.3 ± 3.8	32.7 ± 2.7	26.5 ± 2.1	38.1 ± 3.1	30.7 ± 3.5
IgG2a	18.6 ± 2.5	32.5 ± 2.3	44.0 ± 3.4	37.8 ± 3.4	24.8 ± 2.8	32.3 ± 2.4	31.0 ± 2.4
IgG2b	20.7 ± 1.9	30.2 ± 1.7	41.5 ± 4.1	35.6 ± 2.4	28.8 ± 1.8	36.0 ± 2.6	32.5 ± 2.5
IgG3	29.7 ± 2.6	33.0 ± 2.4	39.4 ± 3.6	36.3 ± 2.6	24.6 ± 3.0	38.5 ± 2.2	34.2 ± 2.8
IgM	32.3 ± 4.2	45.2 ± 3.8	79.5 ± 5.7	53.3 ± 3.7	32.6 ± 3.2	68.3 ± 4.6	42.1 ± 3.9
IgA	8.2 ± 1.2	11.7 ± 1.0	24.1 ± 2.5	19.4 ± 1.3	15.8 ± 1.8	13.7 ± 1.0	11.4 ± 2.2
IgE	6.0 ± 0.2	7.2 ± 0.8	14.6 ± 0.5	7.7 ± 0.6	7.5 ± 0.9	10.7 ± 0.5	7.0 ± 0.7

^a Antisera from immunized mice were obtained, and Ig production was determined by ELISA as described in *Materials and Methods*. Results ($\mu\text{g/ml}$) are expressed as mean $\pm$ SD from three mice.

8B, splenocyte proliferation was remarkably increased in the case of cells obtained from mice injected with rHRF or rHRF plus PV200 Ag compared with cells obtained from mice that did not receive rHRF. In addition, the secreted Ig was measured (Table III). As shown for cell proliferation, Ig production was elevated after *in vivo* injection of rHRF or HRF cDNA compared with the control level.

Discussion

T cell-derived cytokines and CD40L are responsible for T cell-dependent B cells activation, proliferation, and Ig isotype switching. However, some Ags and cytokines derived from non-T cells are known to activate B cells in T cell-independent responses. IFN- γ produced from NK cells enhances IgG3 production, and TGF- β causes IgA secretion from B cells (10). A Toll-like receptor protein, RP-105, can trigger B cell activation without any costimulators (13).

IgE-HRF was originally known as a complete secretagogue for histamine and IL-4 from basophils expressing IgE⁺. However, HRF could increase histamine and IL-4 from IgE⁻ basophils when preincubated with HRF and challenged with anti-IgE Ab (18). Also, HRF did not bind to IgE. Rottlerin, a nonstaurosporine-derived kinase inhibitor isolated from *Mallotus philippinensi*, discriminated between HRF and other IgE-dependent (anti-IgE and Ag) histamine release (19). It had no effect on histamine release induced by anti-IgE, but it enhanced rHRF-mediated histamine release. These results suggest that HRF activates cells by interacting with a specific receptor on the cell surface.

In this paper we identified IgE-HRF as a B cell stimulatory factor from the culture supernatants of LK1 cells that also expressed IL-5 and IFN- γ (20). The effects of rHRF on B cell functions were demonstrated in several aspects. First, rHRF directly enhanced B cell proliferation at a concentration of less than $1\mu\text{g/ml}$. It had strong synergies with the known B cell stimulatory cytokines and anti-CD40 Ab. In the presence of anti-CD40 Ab,

rHRF could activate B cell growth at a concentration of $<10\text{ ng/ml}$. It induced B cell blast formation maximally in the presence of anti-CD40 Ab and anti-IgM Ab. Second, rHRF induced MHC class II expression, a marker for B cell activation (13). Third, it activated NF- κB activity of B cells especially in the presence of anti-CD40 Ab. Finally, it could stimulate *in vitro* and *in vivo* Ig production from B cells. In addition to functional aspects, a binding study of HRF to B cells demonstrated that HRF binds B cells via its own receptor, which is different from IgE as suggested previously (19). These data from studies of B cell proliferation and differentiation clearly demonstrated that rHRF is another B cell stimulatory factor that has synergies with other known B cell activation factors.

Immature B cells, characterized by the expression of mIgM, migrate to the periphery where they transverse a transitional stage to become naive mature B cells (29). CD69 has been known as an earliest leukocyte activation maker. A recent study reported that CD69-deficient mice showed normal functions of T cells, platelets, neutrophils, and eosinophils (28). However, CD69 knockout mice showed a significant increase in the number of pre-B and immature B cells compared with wild-type mice. These results indicate that CD69 plays a critical role in B cell development and early activation. Recombinant HRF increased CD69 expression significantly (Fig. 5), and it affected IgM production the most, especially in the presence of anti-CD40 Ab (Tables I and III). Other differentiation Ags, such as CD23 and CD44 (30, 31), were not affected by rHRF. In addition, its effect on Ig production was partially inhibited by Abs against IL-6 or IL-10, which were induced by rHRF. Based on these results, the effects of rHRF on B cell functions suggest that rHRF induces B cell proliferation more than differentiation into Ig-secreting plasma cells compared with polyclonal B cell activation.

Removal of bacterial endotoxin (LPS) contamination was one of the major concerns for the purification of rHRF. It was also monitored in different ways. First, boiling or Ab treatment completely

FIGURE 8. *In vivo* effects of rHRF on Ig production and cell proliferation. Recombinant HRF (20 $\mu\text{g/injection}$) or PV200 peptide (20 $\mu\text{g/injection}$) was injected into BALB/c mice on days 21, 31, and 41 as described in *Materials and Methods*. A, Antisera from these mice were obtained, and the production of anti-PV200 Ab was determined by ELISA. B, Splenocytes ($2 \times 10^5/\text{well}$) obtained from these immunized mice were restimulated with anti-CD40 Ab (250 ng/ml) for 3 days. Cell proliferation was measured by [^3H]thymidine uptake assay. Data are expressed as the mean $\pm$ SD of triplicate cultures. *, $p < 0.05$ vs all other groups.

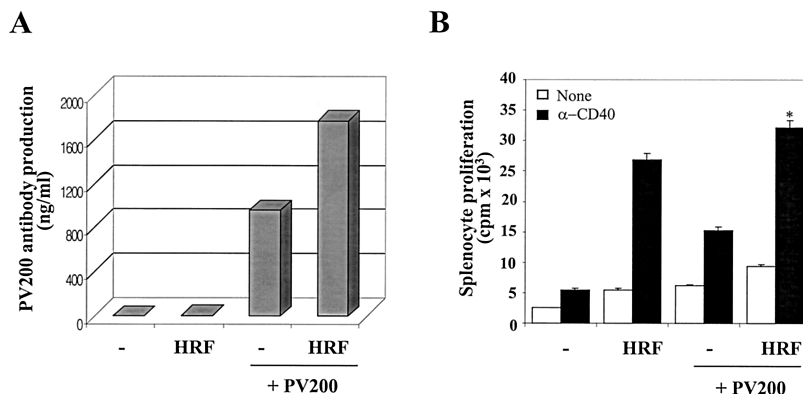


Table III. Ig production from splenocytes derived from immunized mice after restimulation with anti-CD40 Ab^a

Ig Class	Normal		PV200		PV200 + rHRF		rHRF		pcDNA		pcDNA – HRF	
	–	+	–	+	–	+	–	+	–	+	–	+
IgG1	137 ± 14.2	213 ± 12.1	203 ± 12.0	287 ± 14.2	274 ± 13.3	449 ± 24.5	229 ± 14.4	262 ± 12.3	127 ± 11.5	189 ± 10.5	195 ± 14.1	224 ± 21.5
IgG2a	132 ± 11.3	226 ± 14.5	194 ± 11.8	266 ± 12.2	267 ± 15.4	471 ± 23.1	225 ± 12.8	287 ± 14.9	132 ± 10.2	188 ± 8.5	195 ± 10.5	243 ± 14.3
IgG2b	145 ± 14.1	246 ± 24.4	218 ± 13.4	261 ± 13.3	366 ± 18.6	445 ± 21.6	217 ± 11.2	288 ± 13.2	125 ± 13.4	190 ± 10.1	187 ± 9.8	236 ± 18.2
IgG3	95 ± 8.3	218 ± 11.2	205 ± 15.6	290 ± 17.1	238 ± 12.0	390 ± 13.4	225 ± 14.3	266 ± 10.0	127 ± 12.0	188 ± 14.8	194 ± 12.4	250 ± 14.6
IgM	161 ± 15.7	221 ± 10.7	220 ± 10.4	325 ± 21.2	304 ± 20.3	679 ± 28.5	268 ± 16.0	359 ± 43.8	254 ± 22.1	373 ± 21.4	300 ± 10.7	410 ± 25.8
IgA	75 ± 2.4	116 ± 13.4	86 ± 5.6	177 ± 10.9	115 ± 10.6	243 ± 12.7	110 ± 6.5	189 ± 10.4	85 ± 4.3	158 ± 12.6	120 ± 5.8	258 ± 20.4
IgE	45 ± 2.8	46 ± 3.4	44 ± 1.3	44 ± 2.4	46 ± 8.2	46 ± 2.5	46 ± 1.8	46 ± 1.8	47 ± 1.5	44 ± 1.8	47 ± 2.6	51 ± 4.7

^a Splenocytes (5×10^5) derived from immunized mice were treated with anti-CD40 Ab (250 ng/ml) for 3 days. After incubation, culture supernatants were collected and measured by ELISA as described in *Materials and Methods*. Results (ng/ml) are expressed as mean $\pm$ SD of three determinants.

abolished the HRF effects on B cell growth. Second, rHRF from 293 mammalian cells showed the similar B cell growth activity. Third, Ig production induced by LPS or rHRF showed different patterns: rHRF induced higher levels of IgM than LPS did, but to a lesser extent in the cases of IgG and IgA. Fourth, PMB which neutralizes the biological activity of LPS (26, 27), reduced LPS effects on Ig production (Table I) and MHC class II expression (Fig. 5), but did not have any effect on rHRF activity. Furthermore, LPS and rHRF exhibited the different effects on the induction of IL-6 gene expression and NF- κ B activation in the presence of anti-CD40 Ab (Fig. 7). Collectively, the effects of rHRF on B cell functions such as Ig secretion, MHC class II, and gene expression were distinguished from those of LPS.

Histamine itself affects B cell proliferation and differentiation. Histamine induces B cell proliferation in the presence of anti-IgM Ab only when the serum was dialyzed and c-Kit-positive cells were removed (32). It enhances IgE and IgG4, but has no effect on IgG, IgM, or IgA production in the presence of IL-4 or IL-13 (33). Based on the culture conditions and Ig production patterns, it is unlikely that the effects of rHRF on B cell activities in this study are due to the indirect effects of histamine production.

During the process of B cell activation and differentiation, phenotypic and functional changes occur, such as Ig rearrangement and expression of cell surface molecules. These cellular events are integrated by transcription factors, which execute a program of activation and differentiation by regulating gene expression. Among them, PU.1 has been known to regulate various B cell-specific genes, including mb-1, Ig J chain, and Ig κ -chain, by co-operating with other transcription factors, such as Pip and c-Fos (34). NF- κ B is also involved in regulating the Ig light chain gene and B cell activation. In vivo, NF- κ B-null mice have a defect in B cell activation and Ig secretion. Recombinant HRF activated NF- κ B activity and NF- κ B-related cytokine gene expression, especially in the presence of anti-CD40 Ab. Identification of an HRF receptor and receptor-mediated signaling will elucidate the molecular actions of HRF in B cells.

CD40L cooperates with various cytokines to induce B cell activation, proliferation, and Ig isotype switching. CD40L, BAFF, and CD30L are expressed by T cells and are important for the functions of B cells at multiple steps of the T cell-dependent immune response. CD40L counteracts apoptotic signals in B cells after B cell receptor engagement, but BAFF is not able to rescue B cells from anti- μ -mediated apoptosis (12). HRF is expressed in various types of cells, including T cells, monocytes, fibroblasts, and some tumors (14). It is also released from macrophages, which are stimulated with macrophage CSF (35). HRF binding patterns (Fig. 6) indicated that B cells have unidentified HRF-binding molecules that were functionally active in basophils, mast cells, and eosinophils also, suggesting that it may be involved in a broad

spectrum of immune responses. Functionally, HRF has been well known as a critical factor in late phase allergic reaction (15, 16). In addition, our report and other studies (15, 19) have shown that HRF stimulates target cells in the absence of IgE. Taken together, these results indicate that HRF itself functions as an initiator for multi-immune regulators, including B cell activation, mediating the possible interaction between allergic response and Ab production. More dissected studies, including identification of its receptor and possible roles of HRF in B cell apoptosis, are required to understand the HRF-mediated molecular events leading to humoral immune responses.

In this paper we identified the IgE-HRF as a B cell activation factor. IgE-HRF showed synergy with anti-CD40 Ab in B cell proliferation and activation. However, its effects were different from the effects of LPS on Ig production and inhibitor sensitivity.

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