

A ROLE FOR Lys-His-Gly-NH₂ IN AVIAN AND MURINE B CELL DEVELOPMENT

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Received October 26, 1988; accepted April 13, 1989

Lys-His-Gly-NH₂ has been claimed to selectively induce B cell precursors to differentiate into mature B lymphocytes. In the present study, the effects of this tripeptide and a control compound having the reverse sequence (Gly-His-Lys-NH₂) on growth and differentiation of chicken and mouse B cell precursors were investigated. When chicken bone marrow (BM) cells from 15-day-old embryos were treated for 18 hr with either of the tripeptides, the frequency of Bu-1 antigen-bearing cells increased. Moreover, when embryonic bursa cells were stimulated *in vitro* with phorbol myristate acetate, which induces them to proliferate and undergo terminal differentiation into immunoglobulin (Ig)-secreting cells, these compounds caused a 10-fold increase in the number of Ig-secreting cells but did not increase cell proliferation. They had no effect on neonatal or adult bursa cells. Embryonic bursa cells were cultured in the presence of either of the tripeptides and metabolically labeled with [³⁵S]methionine. When immunoprecipitated Ig was analyzed by two-dimensional gel electrophoresis, no differences in μ heavy or λ light chain diversity patterns could be detected, indicating that neither of these compounds enhances Ig diversification. The effect of these tripeptides on murine B cell precursors was assayed in cultures of BM cells depleted of mature B cells by 5-fluorouracil. When precursor cells were incubated without adherent BM stromal cells, they did not respond to the tripeptides. However, after incubation of precursors with adherent stromal BM cells for 2 days, followed by treatment with either of the two tripeptides, differentiation into lipopolysaccharide-reactive mature B cells took place. Incubation of precursors with adherent stromal BM cells in the absence of tripeptides was not sufficient to allow the precursors to complete differentiation. In addition, both tripeptides acted synergistically with interleukin 1 or interleukin 3. In conclusion, these tripeptides seem to enhance precursor B cell differentiation in a lineage-nonspecific manner rather than to function as lineage-specific differentiation hormones. © 1989 Academic Press, Inc.

INTRODUCTION

B lymphopoiesis is associated with particular hemopoietic tissues acting as unique microenvironments in which B cell progenitors are stimulated to grow and differentiate to functional cells (1). In birds, the bursa of Fabricius provides such a microenvironment. The diversification of a single functional germ line V-gene for both the light and the heavy chain occurs by somatic gene conversion-like mechanisms, thus creating the preimmune Ig-receptor repertoire (2). In mammals, however, the B cell system is generated and maintained by continuous differentiation of large numbers

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² Founded and supported by F. Hoffmann-LaRoche & Co., Ltd., Basel, Switzerland.

of precursor cells from stem cells in the bone marrow (BM)³ (3, 4), and the antibody repertoire is created by selecting V, D, and J immunoglobulin (Ig)-gene segments for rearrangement from a relatively large pool (5).

Stromal cells of B lymphopoietic organs may mediate their regulatory effects via direct cell contact and/or by the production of diffusible growth and differentiation factors (6). One such factor from bursal extracts, a tripeptide (Lys-His-Gly-NH₂) termed "bursin," has been suggested to function as a bursal hormone selectively inducing phenotypic differentiation (expression of the Bu-1 alloantigen) in avian precursor B cells from embryonic BM or bursa, but not avian precursor T cells (7, 8). Natural as well as synthetic tripeptides increase levels of cyclic adenosine monophosphate and guanosine monophosphate in the human Daudi B cell line but not in human CEM T cells (9). Similar effects have been detected using the murine B cell line MOPC 315 (9).

In the present study, the capacity of Lys-His-Gly-NH₂ and its reverse structure Gly-His-Lys-NH₂ to sustain B cell precursor differentiation was evaluated with both chicken and mouse cells. No evidence for a specific action was found. However, a nonspecific differentiation enhancing activity was detected with properties similar to those of a previously described tripeptide known to modulate cell growth by facilitating copper uptake in a variety of cell types (10, 11).

MATERIALS AND METHODS

Animals. H.B21 chickens were obtained from the Institute's breeding farm (Gipf-Oberfrick, Switzerland). Female or male BALB/c mice, 11–15 weeks old, were obtained from Harlan OLAC, Ltd. (Blackthorn, Bicester, Oxon, England) or from IFFA CREDO (L'Arbresle, France). The animals were specific pathogen-free and kept in a barrier-sustained unit. Donors for precursor B cells were treated with 5-fluorouracil (5-FU; Sigma No. F-6227, St. Louis, MO) at 150 mg/kg body wt 5 days before BM cells were collected. Female Lewis rats not older than 5 weeks (from the Institute for Biomedical Research, Füllinsdorf, Switzerland) were used to obtain thymus filter cells.

Monoclonal antibodies. Monoclonal antibody L.22 was prepared as previously described and was used to stain BM cells for the Bu-1a marker (12). Monoclonal antibodies M-4 and L-1 (Southern Biological Associates, Birmingham, AL) were used to stain μ and λ chains on the cell surface (13).

Interleukins (IL). Mouse recombinant IL-3 (rIL-3; a gift from Dr. Hajime Karasuyama, Basel Institute for Immunology) was obtained from the supernatant of the transfected mouse B cell myeloma X63/0 (14) and stored at 4°C. Human recombinant IL-1 α (rIL- α) expressed in *Escherichia coli* was purchased from CISTRON Biotechnology (Pine Brook, NJ).

Synthetic peptides. Gly-His-Lys-NH₂ and Lys-His-Gly-NH₂ were synthesized with an Applied Biosystems Model 430A peptide synthesizer, employing the standard solid-phase method on 4-methylbenzhydrylamine resin (15, 16). The peptides were

³ Abbreviations used: BM, bone marrow; Ig, immunoglobulin; 5-FU, 5-fluorouracil; IL, interleukin; rIL, recombinant interleukin; EBSS, Earle's balanced salt solution; FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; PMA, phorbol myristate acetate; LPS, lipopolysaccharide; IMDM, Iscove's modified Dulbecco's medium; DxS, dextran sulfate; PFC, plaque-forming cell(s); aMW, apparent molecular weight.

deprotected and removed from the resin by anhydrous hydrogen fluoride in the presence of anisole (17). Then, the peptides were purified by HPLC on a C18 reverse-phase column (Vydac).

Culture of bursa cells. Single cell suspensions from bursal tissue fragments were prepared as previously described (18). The cells were prepared in cold Earle's balanced salt solution (EBSS) with 1% fetal calf serum (FCS; GIBCO, Grand Island, NY), washed once, and immediately resuspended in culture medium (freshly prepared Iscove's modified Dulbecco's medium (IMDM) adjusted to the correct osmolality for chicken cell cultures: 350–360 mosmol) (19). Culture medium was supplemented with FCS, bovine serum albumin, chicken transferrin, 2-mercaptoethanol (2-ME), and gentamycin as previously described (19). B cells were polyclonally activated with 50 ng/ml of phorbol myristate acetate (PMA, Sigma; dilution made from a stock solution containing 10 mg/ml PMA in absolute ethanol), 50 μ g/ml of lipopolysaccharide (LPS from *Salmonella typhosa*; Difco, Detroit, MI), or 25 μ g/ml of dextran sulfate (DxS; Pharmacia, Uppsala, Sweden). Cells were cultured at 5×10^5 in flat-bottom microtiter plates (Costar No. 3596, 96 wells; Cambridge, MA) in a volume of 0.2 ml. The cultures were incubated for 42 hr at 37°C in 5% CO₂ and 95% air.

Cultures of murine B cell-depleted BM cells. BM cells were depleted of adherent cells by filtration over Sephadex G-10 beads. The method and the preparation of single cell suspensions have been described in detail (20, 21). Mature B cells were eliminated by adsorption on Optilux polystyrene petri dishes (100 \times 15 mm; Falcon No. 1001, Oxnard, CA) coated with 10 ml of monoclonal anti- μ chain antibody (C-2-23; 5 mg protein/ml). The detailed procedure has been published elsewhere (20). B cell-depleted 5-FU resistant BM cells (10^6 in 2 ml of complete RPMI 1640 medium) were conditioned for 2 days on stromal BM cells in Costar six well cluster dishes (Costar No. 3506). Nonadherent cells were removed, and cell clusters composed of adherent and nonadherent cells were dissociated by 15–30 min incubation at 37°C in collagenase solution (collagenase type 2, No. C-6885, Sigma; 0.15% (w/v), in phosphate-buffered saline containing also 1 μ g/ml of DNase) and subsequent filtration over Sephadex G-10. The cells passing through the G-10 column were collected, washed once with cold balanced salt solution (BSS), and resuspended in RPMI 1640 medium supplemented with FCS, antibiotics, and 2-ME. Cells were incubated in microcultures with or without adherent cells at a density of 2×10^4 /0.2 ml. In cultures without stromal cells, the wells had been coated with human fibronectin (25 μ g/ml; 50 μ l/well; 2 hr at 37°C) (22) kindly provided by Dr. Robert Pytela, Basel Institute for Immunology. Interleukins and/or tripeptides were resupplied to the microcultures by replacing one-fourth of the volume with factor-containing medium every 72 hr. Incubation was continued for 7 to 10 days.

Establishment of murine adherent BM cell layers. This method has been described in detail (20, 21). Adherent BM cells were established in two ways: (i) in flat-bottomed Costar microtiter plates by adding to each well 1.5×10^5 BM cells in 200 μ l of IMDM supplemented with 5% FCS (Batch No. 20Q17 45D; GIBCO Europe, Paisley, Scotland), antibiotics (penicillin, streptomycin), and 5×10^{-5} M 2-ME; (ii) in Costar six-well cluster dishes by seeding 2×10^6 BM cells in IMDM supplemented with 5% FCS to each well. Nonadherent cells were removed after 5 days and adherent cell layers were allowed to grow to confluence for 2 weeks.

Biosynthetic labeling of cultured bursa cells. After one wash with warm EBSS, cultured cells were biosynthetically labeled with [³⁵S]methionine (100 μCi/0.2 ml; Amersham, England) for 6 hr using methionine-free Dulbecco's modified Eagle's medium (Amimed, Basel, Switzerland) supplemented with 10% dialyzed FCS. After labeling, the cells were washed once with ice-cold EBSS and lysed with ice-cold lysis buffer (10 mM Tris-HCl, pH 8, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.1% NaN₃). The supernatant and cell lysate from cell cultures were combined and used for immunoprecipitation with protein A-coated Sepharose beads (Pharmacia) and rabbit anti-chicken Ig (7 S fraction; Nordic, Tilburg, The Netherlands) as previously described (23).

Two-dimensional gel electrophoresis. The method of O'Farrell (24) was employed. Immunoprecipitated samples were dissolved in sample buffer (9.5 M urea, 2% ampholine pI range 3.5–10, 5% 2-ME). The gels were processed for fluorography and exposed for 1–7 days on Kodak XR5 film at –70°C (25).

Assessment of functional B cells. Nonadherent cells were collected from maturation cultures, and the frequency of functional LPS-reactive B cells was estimated by limiting dilution analysis in microcultures as previously reported (20, 26). Ig-secreting cells were monitored in a modified protein A plaque assay (20, 21) originally described by Gronowicz *et al.* (27).

RESULTS AND DISCUSSION

Effects of Tripeptides on the Differentiation of B Cells from the Bursa of Embryonic and Newly Hatched Chickens

In a first series of experiments, BM cells from 15-day-old chicken embryos were incubated with graded amounts of Gly-His-Lys-NH₂ or Lys-His-Gly-NH₂, respectively, and the appearance of Bu-la-bearing cells was monitored by staining the cells with monoclonal antibody L.22 directed against the Bu-la marker and a fluorescein-labeled anti-mouse Ig antibody. Control cultures without peptides contained an average of 8 ± 4% Bu-la-bearing cells (mean value from three experiments ± SD). In cultures containing between 10 and 100 μg/ml of either of the two peptides, 10 to 18% of the cells were Bu-la positive. On the other hand, the proportion of surface Ig-bearing cells was not changed, as assessed using monoclonal antibodies M-4 and L-1, which detect μ heavy and λ light chains, respectively (data not shown).

Table 1 shows the effect of either of the two tripeptides on bursa cell cultures collected from chickens 4 weeks after hatching or from 15-day-old embryonic donors. Cell proliferation, as determined by [³H]TdR incorporation, was only marginally increased, regardless of the ontogenetic stage of the cell donor. There was no direct effect on the number of Ig-secreting cells. On the other hand, addition of either of the two tripeptides at a concentration of 50 μg/ml together with PMA or with a combination of PMA, DαS, and LPS resulted in an 8- to 10-fold increase in the number of Ig-secreting cells if the cultures contained embryonic bursal cells. In contrast, no effect was observed when cells from 4-week-old bursa were used. It is noteworthy that the tripeptides had to be continuously present in culture. Preincubation for 30 min was insufficient to increase the number of PFC significantly. These results suggest that both peptides enhance polyclonally induced embryonic bursal cell differentiation into antibody-secreting cells. After hatching, however, bursa cells no longer respond

TABLE I
Effect of Tripeptides on the Proliferation and Differentiation *In Vitro* of Chicken Bursal Cells from 15-Day-Old Embryos and 4-Week-Old Birds

Age of bursal cells	Tripeptide (50 µg/ml)	Control		PMA		PMA + D _x S + LPS	
		³ H]TdR uptake (cpm × 10 ⁻³)	PFCs/culture	³ H]TdR uptake (cpm × 10 ⁻³)	PFCs/culture	³ H]TdR uptake (cpm × 10 ⁻³)	PFCs/culture
4 weeks	0	5.4 ± 0.6	56 ± 24	41.1 ± 1.5	9,568 ± 874	55.6 ± 2.0	22,080 ± 689
4 weeks	Lys-His-Gly-NH ₂	2.8 ± 0.3	168 ± 48	45.1 ± 3.1	10,816 ± 1034	46.9 ± 3.4	24,320 ± 1034
4 weeks	Lys-His-Gly-NH ₂ ^a	3.4 ± 0.3	64 ± 18	50.5 ± 1.3	11,008 ± 958	67.9 ± 1.5	18,688 ± 1351
4 weeks	Gly-His-Lys-NH ₂	2.2 ± 0.4	24 ± 12	34.3 ± 1.1	9,856 ± 1218	64.7 ± 2.3	20,352 ± 2409
4 weeks	Gly-His-Lys-NH ₂ ^a	3.5 ± 0.4	128 ± 18	47.0 ± 2.3	8,608 ± 532	69.1 ± 2.7	20,672 ± 2150
15-day embryo	0	18.6 ± 1.1	24 ± 12	17.6 ± 1.1	256 ± 48	18.3 ± 1.9	484 ± 64
15-day embryo	Lys-His-Gly-NH ₂	23.8 ± 1.5	160 ± 24	20.0 ± 2.3	2,704 ± 344	25.3 ± 2.1	4,800 ± 320
15-day embryo	Lys-His-Gly-NH ₂ ^a	19.7 ± 1.4	12 ± 12	16.9 ± 1.4	376 ± 112	15.8 ± 1.3	642 ± 198
15-day embryo	Gly-His-Lys-NH ₂	25.0 ± 1.0	120 ± 48	16.3 ± 1.7	2,272 ± 138	24.6 ± 2.0	2,624 ± 424
15-day embryo	Gly-His-Lys-NH ₂ ^a	19.9 ± 1.7	24 ± 12	16.3 ± 2.4	488 ± 68	15.8 ± 1.3	512 ± 64

^a Cells were preincubated with tripeptides 30 min on ice and washed and resuspended into fresh culture medium. Results are given as means ± SD of triplicate cultures.



FIG. 1. 2D gel electrophoretic comparison of heavy (μ) and light (λ) chains from immunoprecipitated, reduced Ig produced by embryonic bursal cells cultured with Gly-His-Lys-NH₂, with Lys-His-Gly-NH₂ (bursin), or without tripeptides (control). Isoelectric focusing (pI range 3.5–10) was in the horizontal dimension, cathode on left. The figure shows autoradiographs (5 days exposure) of the gels.

to the tripeptides. Conceivably, the tripeptides act on stages of the B lineage present in the embryonic bursa but absent from the bursa 4 weeks after hatching.

Effects of Tripeptides on Chicken Antibody Diversification

The generation of antibody diversity in the chicken bursa during ontogeny at the protein level was analyzed by 2D gel electrophoresis (19). Protein diversification was found to occur primarily between Days 15 and 18 of embryogenesis (19). In addition, enhancement of Ig secretion in embryonic bursa cell cultures supplemented with tripeptides was observed. To determine if the tripeptides affected Ig-gene diversification, Day 15 embryonic bursal cells were incubated with either of the two tripeptides and labeled with [³⁵S]methionine after 3 days. The results are shown in Fig. 1. Immunoprecipitates of Ig revealed a group of spots of 70–75 kDa apparent molecular weight (aMW), corresponding to μ heavy chains, and a better resolved pattern of spots in the λ light chain region (aMW: 25–29 kDa). Comparison between gels of cells incubated with or without tripeptides indicated that neither of the tripeptides affected the diversity spectra of μ heavy and λ light chains.

Effects of Tripeptides on Murine B Cell Precursors in Adult BM

A subset of BM cells enriched for early, noncycling progenitors (28, 29) can be obtained from adult murine BM after the donors have been treated with 5-FU. Such precursors require interaction with adherent BM stroma cells in order to generate mature B cells after 7–9 days of incubation (21). In order to score B cell-containing cultures, the nonadherent subpopulation was transferred to fresh microcultures, stimulated with LPS, and Ig-secreting cells were assessed as protein A PFC after 4 days.

To investigate whether the effects of the stromal microenvironment could be mimicked by the tripeptides, B cell progenitors were exposed to them in the absence of stromal BM cells. As shown in Fig. 2, no mature, LPS-responsive B cells appeared under these conditions. However, when B cell progenitors were allowed to interact with adherent BM stroma for 48 hr (conditioning of precursors) and incubation was

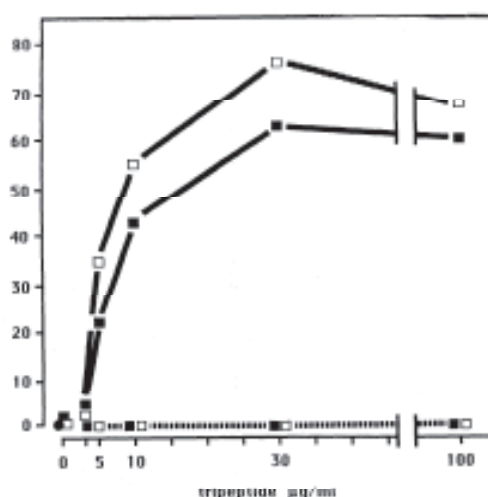


FIG. 2. Generation of LPS-responsive B cells in cultures of B cell-depleted BM precursors collected from mice treated with 5-FU. Precursors either were cultured in the presence of graded doses of Lys-His-Gly-NH₂ (□—□) or Gly-His-Lys-NH₂ (■—■) without adherent BM cells or were first conditioned on an established layer of adherent BM cells for 48 hr and then incubation was continued without stroma cells in medium supplemented with Lys-His-Gly-NH₂ (□—□) or Gly-His-Lys-NH₂ (■—■) or without tripeptide (●). Cultures were scored positive if they contained LPS-reactive cells giving rise to more than five protein A PFC. Twenty microcultures were set up for each concentration of tripeptide (0.2 ml: 2×10^4 cells). One representative experiment is shown.

continued in wells coated with fibronectin and medium supplemented with either of the two tripeptides, there was a dose-dependent increase in the proportion of positive cultures (Fig. 2). When conditioned precursors were cultured in a similar way but without tripeptides, they did not develop into mature B cells. In conclusion, besides the signal given by the tripeptides, an additional signal provided by direct cell-cell contact is required for B cell precursor maturation, which is in accordance with work published previously (30, 31). Precursor cells cultured in insert chambers, separated from a layer of BM stroma by a nucleopore membrane, formed mature B cells only if they had been preconditioned on adherent BM cells and if nonadherent accessory cells had been cultured with stroma cells in the surrounding well (30). The nonadherent accessory cell involved might be a T lymphocyte, since a mixture of dendritic cells and T cells seems to be required in order to complete differentiation of IL-3-dependent precursor B cell clones (32–34).

Conditioned precursors cultured with tripeptides yielded 70–80% positive cultures, while all cultures became positive after the precursors had been allowed to differentiate on BM stroma during the entire culture period (Fig. 2). Although there was a dose-dependent increase in the number of LPS-induced PFC obtained from positive cultures, it never reached the extent observed after continuous coculturing of precursor cells with BM stroma cells (Fig. 3). This is consistent with the finding that supernatants conditioned by cloned stroma cells support neither the short-term nor the long-term growth of B cells with the same growth kinetics as those of cells growing in direct contact with an uncloned adherent cell layer (6). Therefore, additional factors might

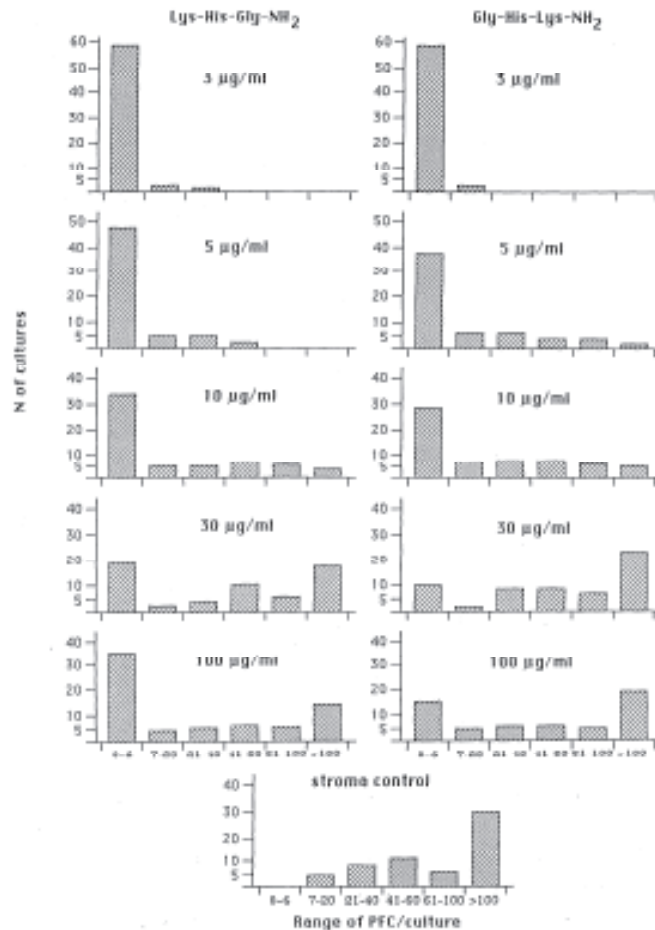


FIG. 3. Range of antibody-secreting cells (protein A PFC) arising in microcultures after LPS stimulation. The cultures contained murine BM precursors conditioned on BM stromal cells for 48 hr and then cultured in the presence of tripeptides. Stromal control: precursors continuously cultured with adherent BM stromal cells prior to LPS stimulation. Each group consists of 60 microcultures. Otherwise same conditions as those in Fig. 2.

be involved. On the basis of the observation that the panspecific interleukins IL-1 and IL-3 partially support final differentiation of B cell precursors conditioned on BM stroma (30), tripeptides were tested in combination with IL-1 and/or IL-3.

Synergism with IL-3 and IL-1

The results of these experiments are summarized in Fig. 4. Neither of the interleukins alone allowed significant maturation of B cell progenitors. However, combinations of the tripeptides with either IL-1 or IL-3 supported terminal differentiation of preconditioned progenitors more efficiently than the mixture of IL-3 and IL-1.

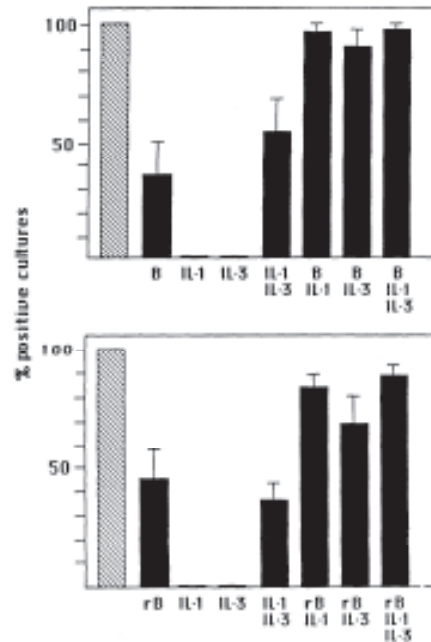


FIG. 4. Tripeptides potentiate the effects of IL-3 and IL-1 on preconditioned progenitor B cells (5-FU insensitive, mature B cell-depleted BM cells). Each bar represents the arithmetic mean (\pm SD) of three independent cell culture experiments. Each experimental group consists of 20 microcultures. Murine rIL-3: from supernatant of transfected B cell myeloma X63/0, 3%; human rIL-1 α expressed in *E. coli*, 3 U/ml. Otherwise same conditions as those in Fig. 2. \square , Precursors cultured on stromal BM cells all the time. \blacksquare , Precursors conditioned on stromal BM cells for 24 hr. B, Lys-His-Gly-NH₂ (bursin); rB, Gly-His-Lys-NH₂ (reverse homolog of bursin).

Taken together, these findings suggest that Lys-His-Gly-NH₂ (bursin) modulates the maturation of subsets of avian and murine B cell precursors. The reverse homology, however, was as active as the molecule present in the bursa of Fabricius. It is possible that both tripeptides, by binding copper to histidine, promote copper uptake. As shown for Gly-His-Lys-NH₂ from rat liver an increase in copper uptake leads to enhanced growth of a variety of cells *in vitro* (10, 11). In the experiments with chicken cells reported here we found no evidence for an increase in cell proliferation. However, it is possible that terminal differentiation of late postmitotic precursor B cells (4) could be enhanced by such a mechanism and this may represent one of the functions of the bursal or BM microenvironment. Finally, elevation of cAMP or cGMP, which has been reported to be induced in Daudi cells by Lys-His-Gly-NH₂ and to a much lesser degree by Gly-His-Lys-NH₂ (9), may also play a role. Our data clearly exclude that either of these tripeptides acts as a lineage-selecting differentiation hormone or as a regulator of V-gene diversification.

ACKNOWLEDGMENTS

We thank Bente Walch, Anita Söderberg, Lotte Kuhn, and David Avila for excellent technical assistance; Drs. Olli Vainio, Ivan Lefkovits, and Ton Rolink for critical comments on this manuscript; Dieter Büschten and Hans Spultinger for artwork, and Christina Brügger for expert editorial assistance.

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