Interleukin-5 Is an Autocrine Growth Factor for Epstein-Barr Virus-Transformed B Lymphocytes

By Michael A. Baumann and Cassandra C. Paul

Because of the recent finding that interleukin-5 (IL-5) is produced by Epstein-Barr virus-transformed B lymphocytes (EBV-B cells), we performed studies to ascertain whether EBV-B cells might use IL-5 by an autocrine mechanism. EBV-B cells known to be IL-5 producers were capable of responding to addition of exogenous IL-5 by dose-related augmented proliferation. The addition of a neutralizing anti–IL-5 antibody reduced these effects and also dose-dependently inhibited proliferation and reduced viability of unsupplemented EBV-B cells, having a maximum effect at about 120 hours. In contrast, no stimulatory effect of IL-5 was noted on Burkitt's lymphoma cell lines, nor were these lines growth-inhibited by anti–IL-5 antibody. With biotinylated IL-5, (b-IL-5) second labeling with streptavidin-FITC, and flow cytometric analysis, binding of IL-5 to EBV-B cells cultured in fresh medium was demonstrated and could be competed for by excess unlabeled IL-5, suggesting the presence of IL-5–specific binding sites. Binding of IL-5 was reduced on cells cultured for longer periods before study but could be restored by extensively washing cells before labeling them with b-IL-5, suggesting that surface binding sites had become occupied by endogenously produced IL-5. These findings support a role for IL-5 in autocrine support of EBV-B cell growth.

INTERLEUKIN-5 (IL-5) was originally characterized in murine systems, where it was produced by T lymphocytes and functioned to promote B-cell growth and differentiation and differentiation of eosinophil progenitors.[1,2] The murine IL-5 gene was used to isolate and clone the human IL-5 gene,[2-4] permitting recombinant production of human IL-5. Human IL-5 has been shown to promote eosinophil growth and differentiation,[5] but evidence of an effect of IL-5 on human B cells has not previously been convincingly demonstrated.[6]

Recently, we showed that normal human B cells that have been transformed by the Epstein-Barr virus (EBV) constitutively produce IL-5.[7] We now report evidence that IL-5–producing EBV-transformed B cells (EBV-B cells) specifically bind IL-5, are growth-inhibited by neutralizing anti–IL-5 antibody, and are capable of responding to IL-5 by augmented proliferation. Thus, EBV-transformed B cells probably produce and use IL-5 in an autocrine fashion.

MATERIALS AND METHODS

IL-5 and antibodies. Immunoaffinity-purified recombinant human IL-5 derived from the NS-1 murine myeloma cell line was a gift from Dr Satwant Narula (Schering-Plough Research, Bloomfield, NJ). The neutralizing, monoclonal, rat IgG2a anti-human IL-5 antibody 39D10[8] was a gift from Dr John Abrams (DNAX, Palo Alto, CA). Monoclonal, rat IgG2a anti-mouse macrophage antibody (hybridoma line 158.2), used as an isotype-matched control, was obtained from the American Type Culture Collection (ATCC). Both antibodies were purified from hybridoma culture supernatants by two 50% ammonium sulfate precipitations followed by extensive dialysis against phosphate-buffered saline (PBS). Antibody was quantified after purification by enzyme-linked immunosorbent assay (ELISA).

Biotinylation of IL-5. Recombinant human IL-5 was extensively dialyzed against 0.1 mol/L $Na2CO_3$, pH 8.4, in preparation for biotinylation. One milligram of IL-5 was reacted with 80 μL N-hydroxysuccinimidobiotin (BioRad) in 1 mL of 0.1 mol/L $Na2CO_3$, pH 8.4, buffer for 4 hours in the dark at room temperature. The reaction was stopped by addition of sufficient Tris-glycine buffer to constitute 0.2 mol/L at pH 8.5. The resultant mixture was passed over a G-25 (PD10) column. Elution and storage buffer used was 0.2 mol/L Tris-glycine, pH 8.5. Biotinylated IL-5 (b-IL-5) was assayed for bioactivity using the TF-1 cell line (a gift from Dr T. Kitamura), as previously reported.[8] Activity was more than 70% of unlabeled rhIL-5.

EBV-B cell lines. EBV-B cell lines arose spontaneously from suspension cultures of peripheral blood mononuclear cells (PBMNC) of persons who had previously been infected by EBV. All lines have become monoclonal in long-term culture, as assessed by immunoglobulin gene rearrangement study. Details of isolation of these cell lines were described previously.[7,9] All cell lines studied have been verified to be constitutive producers of IL-5 by Northern analysis for IL-5 mRNA and by immunoblotting of culture supernatants.[7] Five distinct cell lines were used for these studies and are identified by laboratory codes: 600, 605, 720, 817, and 300.

Burkitt's lymphoma cell lines. The Daudi and Ramos cell lines were obtained from the ATCC and as a gift from Dr William Sugden, Madison, WI, respectively. Both have the t(8;14) chromosomal rearrangement involving the *c-myc* oncogene typical of Burkitt's lymphoma. The Daudi cell line is EBV-infected. Ramos cells do not contain the EBV genome. The BJAB cell line was obtained from Dr Sugden. Although this EBV^- B-lymphocyte line was established from a patient with Burkitt's lymphoma,[10] the cells do not have a translocation involving the *c-myc* oncogene. They contain a t(10p;11), t(10q;13) rearrangement.[11]

Effects of IL-5 and anti–IL-5 antibody on B-cell growth in vitro. Cultures of four EBV-B cell lines were initiated at 5×10^3 cells per milliliter in 25-cm^2 tissue culture flasks in RPMI medium containing 1% Nutridoma HU serum-free supplement (Boehringer Mannheim, Indianapolis, IN) and 2 mmol/L L-glutamine. To parallel cultures were added increasing concentrations of rhIL-5, 5 μg/mL anti–IL-5 Ab, or 5 μg/mL isotype matched irrelevant Ab as control. Cultures were incubated at 37°C and were fed by replacement of one third medium every 72 hours. Replacement medium for the supplemented cultures contained the same concentration of rhIL-5 or antibody. Serial assessment of cell number was by Coulter counting of an aliquot of cultures, and assessment of viability was by trypan blue dye exclusion. Similar studies were

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performed on Burkitt's lymphoma cell lines, as characterized above.

Time and concentration studies of anti–IL-5 antibody on growth of EBV-B cells. Four EBV-B cell lines were cultured at 5×10^3/mL in replicates of four in 96-well tissue culture plates, alone or with addition of increasing concentrations of the neutralizing anti–IL-5 antibody 39D10[8] or irrelevant, isotype-matched antibody as control. Cells were cultured in serum-free medium as described above for 72, 96, 120, 144, and 168 hours before addition of 1 μCi ^{3}H-thymidine and culture for 18 hours longer. Cells were then harvested for determination of relative rate of DNA synthesis by liquid scintillation counting. Similar studies were performed on the three Burkitt's lymphoma cell lines.

Assessment of IL-5 binding by flow cytometry. EBV-B cells were cultured in complete Iscove's medium for varying time periods before study. All cultures were verified to be >95% viable by trypan blue exclusion at the time of analysis. Cells were washed, and aliquots of 1×10^6 were incubated for 30 minutes at room temperature in a minimum volume of Iscove's medium containing 1% fetal bovine serum (FBS) and 1 μg b-IL-5. The cells were then washed and reincubated for 30 minutes at 4°C with 10 μL of 10 μg/mL FITC-streptavidin (GIBCO BRL, Gaithersburg, MD) in PBS containing 1% FBS. The cells were again washed and resuspended in 1 mL PBS containing 1% FBS and 0.01% sodium azide for analysis on a Becton Dickinson FACscan flow cytometer. Data were collected under logarithmic gain with photomultiplier tube (PMT) thresholds set by unlabeled cells. To control for nonspecific binding, samples were run that were labeled only with FITC-streptavidin or with b-IL-5 to which a 100-fold excess of unlabeled IL-5 had been added before second labeling.

RESULTS

Effect of rhIL-5 and anti–IL-5 antibody on proliferation of EBV-B cell lines. All four EBV-B cell lines demonstrated dose-dependent augmentation of proliferation by IL-5 (Fig 1). Addition of neutralizing anti–IL-5 antibody inhibited proliferation of all four cell lines. This inhibition was not observed in response to addition of irrelevant antibody (Fig 1). In addition, cell viability, as assessed by trypan blue dye exclusion, was adversely affected by anti–IL-5. Cells typically remained 90% to 95% viable alone or with IL-5 supplementation. Viability decreased to 50% to 60% by 8 to 10 days in cultures supplemented with anti–IL-5, but remained 90% to 95% in cultures supplemented with irrelevant antibody (data not shown). Augmentation of proliferation due to exogenous IL-5 was prevented by addition of anti–IL-5 antibody (Fig 2). In contrast, proliferation of the three Burkitt's lymphoma cell lines was not appreciably affected by addition of IL-5 (Fig 3).

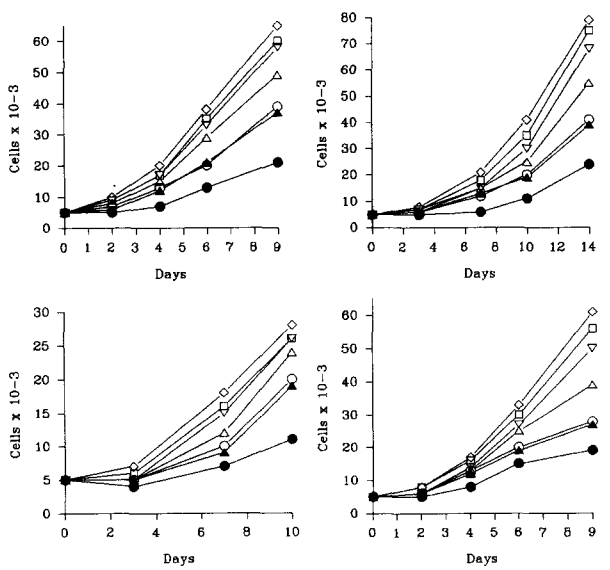

Fig 1. Effect of exogenous IL-5 or anti–IL-5 antibody on proliferation of four EBV-B cell lines. Clockwise from top left: cell lines 600, 605, 720, and 817. Cells alone (○); cells with 5 U/mL IL-5 (△); cells with 10 U/mL IL-5 (▽); cells with 20 U/mL IL-5 (□); cells with 40 U/mL IL-5 (◇); cells with 5 μg/mL anti–IL-5 antibody (●); cells with 5 μg/mL isotype-matched irrelevant antibody (▲).

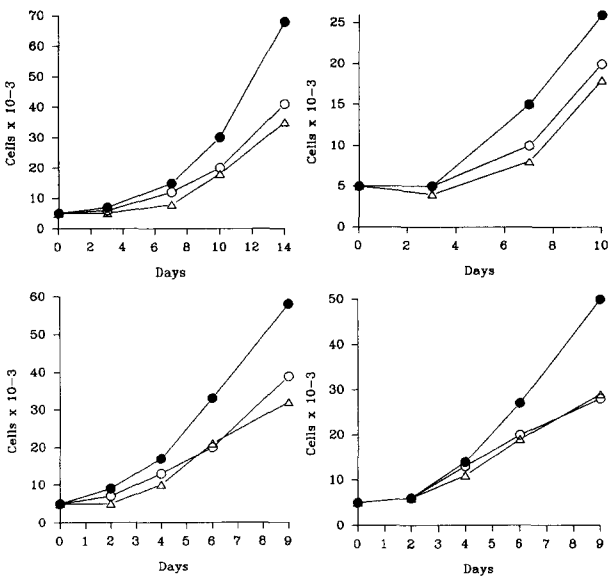

Fig 2. Inhibition by anti–IL-5 antibody of IL-5–enhanced proliferation of four EBV-B cell lines. Clockwise from top left: cell lines 605, 817, 720, and 600. Unsupplemented cells (○); cells with 10 U/mL IL-5 (●); cells with 10 U/mL IL-5 and 10 μ/mL anti–IL-5 antibody (△).

Time and concentration effects of anti–IL-5 antibody on EBV-B cell growth. All four EBV-B cell lines were markedly inhibited by anti–IL-5 antibody in a dose-dependent manner. No inhibition was observed in response to addition of equal concentrations of irrelevant, isotype-matched antibody. Maximum effect was apparent at 120 hours of culture (Fig 4), and significant inhibition was evident by 96 hours (Fig 4E). Proliferation of the Burkitt's lymphoma cell lines was not inhibited by anti–IL-5 antibody (Fig 3D).

IL-5 binding studies. Binding of b-IL-5 was demonstrable on all four EBV-B cell lines studied, and specificity of binding was demonstrated by competitive inhibition of binding of b-IL-5 by a 100-fold excess of unlabeled IL-5 (Fig 5). Increasing the time of cell culture after washed cells were placed into fresh medium before binding study resulted in reduction in detectable binding, and binding was essentially undemonstrable by 72 hours of culture. To ascertain whether this inhibition might be due to occupation of IL-5 binding sites by endogenously produced IL-5, aliquots of 3-day cultures were studied with and without extensive washing of cells (six times) in PBS containing 1%

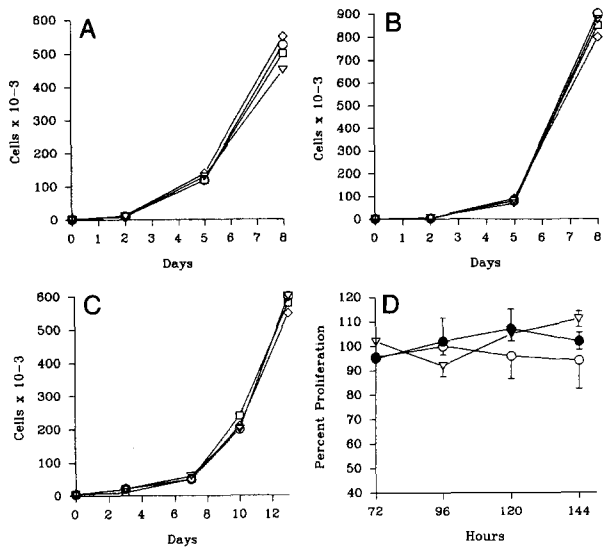

Fig 3. Lack of effect of IL-5 or anti–IL-5 on proliferation of Burkitt's lymphoma cell lines. Proliferation of Ramos (A), Daudi (B) and BJAB (C) cells alone (○), or with 10 U/mL IL-5 (▽), 20 U/mL IL-5 (□) or 40 U/mL IL-5 (◇). (D) Uptake of ^{3}H-thymidine by Ramos (▽), Daudi (○) and BJAB (●) cells incubated with 100 μg/mL neutralizing anti–IL-5 (39D10). Results graphed as the mean ± SD of replicates of four, expressed as a percentage of the proliferation of cells not incubated with anti–IL-5.

FBS. Increased binding of IL-5 was observed on the extensively washed cells, suggesting that endogenously produced IL-5 had occupied surface binding sites (Fig 5). Failure of washing to restore IL-5 binding to the levels observed at 24 hours suggests the possibility that down regulation of surface receptor number may occur in response to continued exposure to endogenously produced IL-5.

DISCUSSION

These studies clearly show that EBV-B cells that constitutively produce IL-5 are also capable of specifically binding IL-5, respond to IL-5 by augmented proliferation in culture, and are growth-inhibited by neutralizing anti–IL-5 antibody. Consideration of these results in comparison with those of previously reported studies suggests possible reasons for the earlier lack of demonstration of IL-5 effects on human B cells.

Although two early studies using crude *Xenopus* oocyte or COS cell supernatants containing IL-5 claimed augmentation of immunoglobulin production by human B cells, larger studies using recombinantly produced human IL-5 could not confirm these results or demonstrate B-cell growth factor activity in other standard assays.[2,6] Specifically, rhIL-5 did not augment proliferation of human tonsillar or human PB B cells costimulated with anti-μ or phorbol myristate acetate (PMA). No activity for IL-5 was observed in a restimulation assay in which B cells were first activated with either *Staphylococcus aureus* Cowan 1 (SAC), a mixture of phorbol dibutyrate and ionomycin, or anti-μ. Neither did human IL-5 increase production of IgG or IgM by purified fresh B cells or of the EBV-B cell line CESS in a restimulation assay with SAC or PMA. Studies of B-cell chronic lymphocytic leukemias and the human lymphoma cell lines L4 and HBF1 also failed to show a response to IL-5.

Several explanations are possible for discrepancies between our results and those of previous studies of human B cells. Of all the materials previously studied, only one study of an EBV-infected cell line was reported, and only immunoglobulin production was assayed, rather than proliferative response.[6] In addition, the cell line used (CESS) was mycoplasma-infected at the time of study,[12] which could have affected results. More important, it was not appreciated earlier that human B cells may be capable of producing IL-5, which could interfere with detection of effects of the addition of exogenous IL-5 under many test conditions. This was amply demonstrated by our IL-5 binding studies, which showed that specific binding of exogenous IL-5 to EBV-B cells is virtually undetectable after as little as 72 hours of culture owing to occupation of binding sites by endogenously produced IL-5. Determination of the relation of the putative receptors detected by these studies to those recently described on human eosinophils[13,14] will require

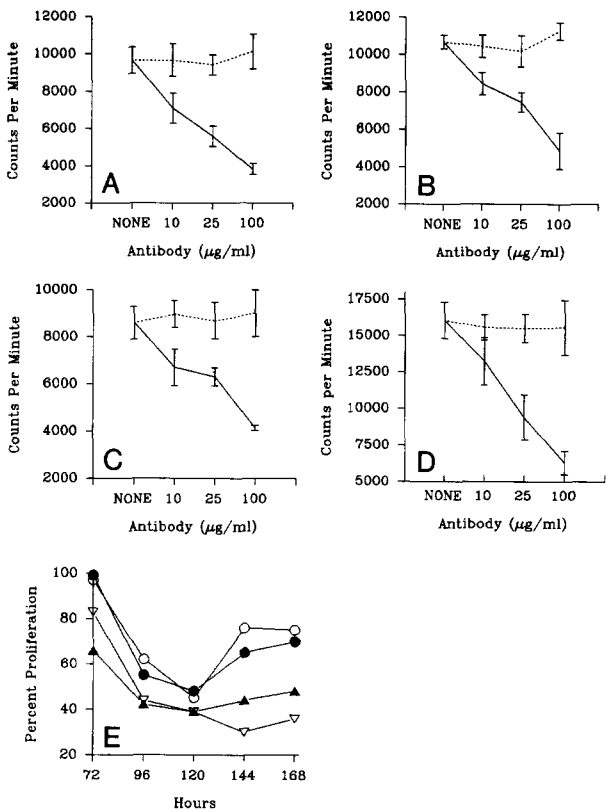

Fig 4. Effects of incubation of four EBV-B cell lines with increasing concentrations of 39D10 neutralizing anti–IL-5 antibody (—) or irrelevant, isotype-matched antibody (···) for 144 hours. Cell lines: 817 (A), 600 (B), 817 (C), 300 (D). Time course of inhibition by 100 μg/mL anti–IL-5 antibody of growth of the four EBV-B cell lines, represented as a percentage of ^{3}H-thymidine incorporation by unsupplemented cells (E). Results are graphed as the mean of four replicates. Error bars are omitted for clarity. Maximum inhibition was observed at approximately 120 hours.

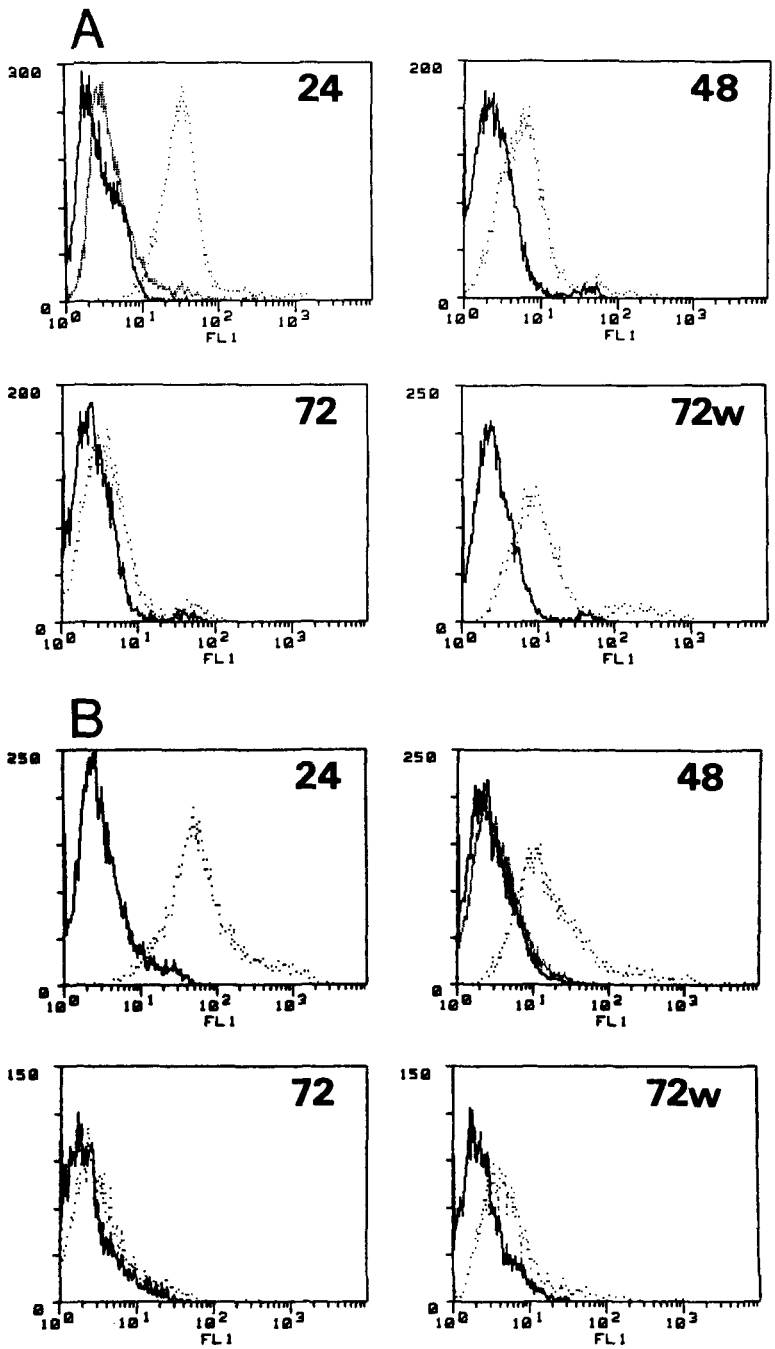

Fig 5. Binding of b-IL-5 to EBV-B cells. Streptavidin-FITC alone (—); 1 μg b-IL-5 (· · · ·); 1 μg b-IL-5 + 100 μg unlabeled IL-5 (···). Representative data from the study of two EBV-B cell lines (A, 605; B, 600) are shown at 24, 48, and 72 hours of culture. Similar results were obtained from four distinct cell lines. Competitive inhibition of b-IL-5 binding by 100-fold excess of unlabeled IL-5 is shown at 24 hours for cell line 605 and at 48 hours for cell line 600. Binding of b-IL-5 at 72 hours was restored after extensive washing of the cells (72 w).

radioligand studies suitable for Scatchard analysis and receptor cross-linking experiments.

Autocrine mechanisms are believed to contribute to perpetual proliferation of EBV-B cells in vitro.[15,16] This has been suspected in part because of the poor growth and viability of EBV-B cells when cultured at very low densities.[16] We theorized that an effect of exogenous IL-5 on the growth of IL-5–producing EBV-B cells would be most apparent on cultures initiated at low density because suboptimal amounts of endogenously produced cytokine might be present under these conditions. Our results support this idea. The relative importance of IL-5 in comparison to other substances that have been proposed as autocrine mediators of EBV-B cell growth, such as soluble CD23[15] and IL-6,[17] remains to be studied. Such studies will be important because of the role of EBV in the pathogenesis of B-cell neoplasms complicating conditions of severe immune suppression.[18-20]

We were not able to demonstrate any effect of IL-5 or anti–IL-5 on proliferation of either EBV-infected or noninfected Burkitt's lymphoma cell lines. Presumably, the events responsible for development of frank malignant growth

characteristics of these cells provide IL-5 independence. In studies of several non–EBV-infected human B-cell lines (including Ramos and BJAB), we have not found evidence of IL-5 production by immunoblotting techniques sensitive enough to detect IL-5 routinely in culture supernatants of EBV-B cells (data not shown). Thus, we have not yet found evidence that IL-5 production and/or response may occur in non–EBV-infected human B-cells. Because EBV-infected normal B cells adopt a phenotype characteristic of B-cell activation,[15] future work will address whether IL-5 production or response may be inducible in noninfected B cells by activation stimuli other than EBV. Continued study of these events should disclose much about the role of IL-5 in human lymphohematopoietic growth regulation.

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