Keratinocyte Derived T-Cell Growth Factor (KTGF) Is Identical to Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)

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Keratinocyte derived T-cell growth factor was initially described as a product of cultured neonatal keratinocytes and keratinocyte cell lines that induced the proliferation of HT-2 cells, a murine T-cell line that responds to IL-2 and IL-4 by incorporating 3H-Thymidine. Subsequently, KTGF has been purified to high specific activity and found to be distinct from IL-2 and IL-4 by a variety of biochemical, immunologic, and immunochemical criteria. Because it was found that certain HT-2 cell lines also proliferated in response to GM-CSF, the present study asked whether KTGF was related to GM-CSF. In this study, we demonstrate that antibodies to recombinant murine GM-CSF completely neutralize the capacity of KTGF to induce HT-2 proliferation without interfering with IL-2 or IL-4 induced HT-2 proliferation. Furthermore, poly-A+ RNA homologous to murine GM-CSF cDNA as judged by S1 nuclease analysis was detected in Pam 212 cells, and protein serologically homologous to GM-CSF was found in Pam 212 conditioned medium. We conclude that KTGF is identical to GM-CSF. The T-cell activating properties of GM-CSF require further exploration. *J Invest Dermatol 91:185–188, 1988*

Keratinocyte derived T-cell growth factor, or KTGF, was initially described as a product of neonatal murine keratinocytes as well as the neonatal keratinocyte cell line Pam 212, which could support the growth and proliferation of a cell line previously considered to be dependent on IL-2 for growth [1]. Shortly after this report, it became clear that while IL-2 was an autocrine growth factor for certain T cells, a factor previously known as B-cell stimulatory factor 1 (BSF-1) and now called IL-4, mediated the autocrine growth of a subset of T helper cells [2,3]. Like IL-2, IL-4 was capable of maintaining the growth of cell lines previously used as bioassays for IL-2, including HT-2 [3,4] and CTLL-2 [5]. Unlike IL-2, IL-4 has been shown to act on non-lymphoid cells such as mast cells and certain myelomonocytic cell lines, and appears to have a broader spectrum of biologic activities [6]. KTGF was purified to a high specific activity and was compared biochemically and biologically to IL-2 and IL-4 [7]. Unlike IL-2, even highly purified KTGF failed to induce proliferation of CTLL-2 cells. While the pI and RP-HPLC profile of IL-2 and KTGF were similar, KTGF differed from IL-4 by both these criteria. Finally, monoclonal antibodies, which neutralized the activities of IL-4 (11B11) and IL-2 (S4B6), had no effect on the activity of KTGF on HT-2 cells [7]. By these criteria, it was concluded that KTGF was distinct from IL-2 and IL-4.

Recently it was reported that recombinant T-cell granulocyte macrophage colony stimulating factor (GM-CSF) could induce the growth and proliferation of certain T-cell lines [8]. We have also demonstrated that human keratinocytes produce GM-CSF after stimulation with IL-1 [10]. Because Pam 212 cells produce IL-1 activity constitutively [1], we reasoned that such cells should also produce high levels of GM-CSF. In the present study, specific antibodies and cDNA probes were used to test the relationship between KTGF and GM-CSF.

MATERIALS AND METHODS

Cell Lines and Assays A subline of HT-2 cells, which responds to GM-CSF, IL-2, and IL-4 [8], was maintained as previously described. Assay conditions were identical to those previously described [8]. Pam 212 cells were obtained from Dr. Stuart Yuspa (National Cancer Institute) and maintained as previously described [11].

Cytokines Purified recombinant human IL-2 was the gift of Dr. Richard Robb, DuPont laboratories (Glenolden, PA). Natural murine IL-4 purified by RP-HPLC and monoclonal antibody affinity chromatography [12] was the gift of Dr. Junichi Ohara, N.I.H. Murine rGM-CSF was obtained from conditioned medium of COS-7 cells transfected with the pCD-GM-CSF expression vector.

Antibodies Goat anti-murine GM-CSF was provided by Dr. Jolanda Schreur (DNAX Research Institute). This reagent was purified from the serum of goats immunized with recombinant non-glycosylated murine GM-CSF by affinity chromatography, and has been shown to neutralize the activity of both recombinant and T-

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Abbreviations:
BSF-1: B-cell stimulatory factor 1
cDNA: complementary deoxyribonucleic acid
GM-CSF: granulocyte macrophage colony stimulating factor
IL-N: Interleukin-N
IM: instant milk
KTGF: Keratinocyte derived T-cell growth factor
mRNA: messenger ribonucleic acid

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cell derived GM-CSF on HT-2 cells [8]. The antibody was used at a dilution of 1 : 1000 for neutralization studies.

S-1 Nuclease Analysis of GM-CSF mRNA The plasmid pcD-mGM-CSF [14] was linearized with Eco RV at position 434 of the GM-CSF insert. The DNA was end-labeled with 32-P-ATP and T4 polynucleotide kinase. Hybridization and S-1 nuclease treatment were performed as described previously [13]. Briefly, approximately 10^4 cpm of denatured probe was hybridized with 10 μg poly A+ mRNA from PAM 212 cells in 15 μl of hybridization buffer (80% formamide, 0.2M PIPES, pH 6.4, 2 M NaCl, 5 mM EDTA) at 50° Centigrade for 16 h. The reaction was diluted with 150 μl cold S-1 nuclease digestion buffer (0.3M sodium acetate, pH 4.5, 2.5 M NaCl, 10 mM ZnC12) containing 100 units/ml S-1 nuclease and incubated at 35° for 45 min. The S-1 nuclease resistant products were run on a denaturing 7M urea 5% polyacrylamide gel. Natural GM-CSF mRNA protects a DNA fragment 434 nucleotides long.

Immunoblot Analysis Serum free conditioned medium from confluent cultures of Pam 212 was concentrated tenfold as described [7]. Medium (0.005 ml) was spotted on Nitrocellulose paper (0.45 um, BioRad Laboratories, Richmond, CA) and allowed to air dry. Recombinant GM-CSF (Genzyme) and concentrated Dulbeccos MEM (Flow Labs) were spotted on adjacent areas of the nitrocellulose paper for positive and negative controls, respectively. The nitrocellulose was incubated to 3 h at room temperature in blocking buffer (NaCl 150 mM, Tris HCl 20 mM pH 7.4) with instant milk (IM) 5% (wt/vol). The blot was washed twice with blocking buffer and then incubated overnight in goat polyclonal anti-GM-CSF antibody diluted 1 : 100 in blocking buffer with 1% IM. The blot was washed three times with blocking buffer and incubated for 1 h with rabbit anti-goat IgG coupled with horseradish peroxidase (Kirkegaard and Perry, Gaithersburg, MD) diluted 1 : 500 in blocking buffer. The blot was washed twice with blocking buffer and incubated for 10–45 min in horseradish peroxidase color development solution (BioRad). The blot was then dried, scanned with a computerized two-dimensional gel scanner (Microscan 1000, Technology Resources, Nashville, TN), and photographed.

RESULTS AND DISCUSSION

Recently, specific monoclonal antibodies, which neutralize murine IL-2 (54B6) and IL-4 (11B11), have been generated [2,12]. In addition, we have reported that a goat antibody to recombinant murine GM-CSF neutralizes GM-CSF activity on HT-2 cells [8]. Previously, it was demonstrated that neither S4B6 nor 11B11 could neutralize KTGF activity [7]. We therefore attempted to neutralize the activity of KTGF on HT-2 cells with the antibody to GM-CSF.

Figure 1 indicates that recombinant IL-4, IL-2, GM-CSF, and RP-HPLC purified KTGF induce proliferation of HT-2 cells in a dose dependent fashion. A 1 : 1000 dilution of anti-GM-CSF antibody completely neutralizes the proliferation of HT-2 cells induced by rGM-CSF and KTGF, but does not affect (or non-specifically enhances) HT-2 proliferation induced by rIL-2 and rIL-4. This indicates that this dilution of antibody is not non-specifically cytotoxic to HT-2 cells. These data strongly suggest that KTGF is serologically related to GM-CSF.

The characterization of the murine chromosomal gene for GM-CSF using a murine GM-CSF cDNA derived from a cloned T lymphocyte cDNA library was reported previously [14]. Figure 2 shows an S1 nuclease analysis of poly-A+ RNA from Pam 212 cells using this cDNA. *Lane 1* shows that Pam 212 cells contain an RNA species that protects a DNA fragment 434 nucleotides long (see "Materials and Methods"), indicating that over this span of nucleotides, Pam 212 GM-CSF mRNA and T-cell derived GM-CSF cDNA are completely homologous. As a positive control *(lane 2)*, total cellular RNA from COS monkey cells transfected with the pcD-mGM-CSF plasmid was used in a parallel hybridization. These GM-CSF transcripts initiate from the SV40 promoter 200 base pairs upstream of the cDNA. When this mRNA is hybridized with the DNA probe, a longer protected fragment is produced compared with natural GM-CSF mRNA, reflecting the additional segment of

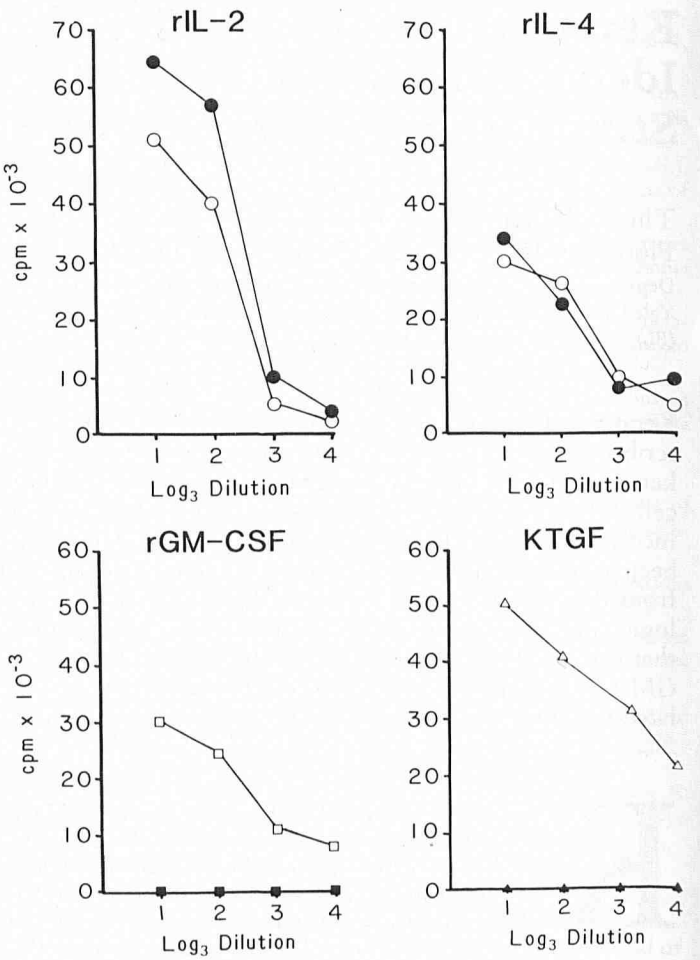

Figure 1. Antibodies to rGM-CSF block KTGF induced proliferation of HT-2 cells. rIL-2 (10U/ml, *Top left, open circles)*, purified IL-4 (20U/ml, *Top right, open circles)*, rGM-CSF (10U/ml, *Bottom left, open squares)*, and RP-HPLC purified Pam 212 conditioned medium *(Bottom right, open triangles)*, were tested in serial dilutions for their capacity to induce HT-2 cell 3H-Thymidine incorporation. A 1 : 1000 dilution of anti-GM-CSF antibody was added to an identical titration curve for each cytokine (represented by the *solid symbol curve* in each graph). The antibody neutralized KTGF and GM-CSF HT-2 proliferation completely without affecting IL-2 or IL-4 proliferation of HT-2 cells.

RNA arising from the vector sequences. The shorter fragments seen in the COS sample represent minor S-1 nuclease digestion products of the RNA/DNA hybrids. *Lane 3* includes no RNA, and *lane 4* includes molecular weight markers.

The immunoblot procedure was used to further confirm the presence of GM-CSF in Pam 212 conditioned medium. As shown in Fig 3, the immunoblot reveals the presence of GM-CSF antigen in Pam 212 conditioned medium. Similar results were obtained in the Western blot of Pam 212 conditioned medium.

We have presented evidence that antibodies to recombinant GM-CSF inhibit the activity of KTGF, as defined by the capacity of Pam 212 and normal neonatal keratinocyte conditioned medium to induce the proliferation of certain sublines of the factor-dependent T-cell line HT-2. We have previously demonstrated that both recombinant GM-CSF and GM-CSF derived from a cloned T helper cell induce proliferation of some but not all HT-2 cells, while neither induces proliferation of CTLL cells [8]. Because the capacity of KTGF to induce HT-2 but not CTLL cell proliferation distinguished it from both IL-4 and IL-2, the possibility that KTGF was related to GM-CSF seemed likely. We have also demonstrated that a mRNA homologous to T-cell GM-CSF cDNA could be identified in Pam 212 cells. Finally, we have shown immunoreactive antigen

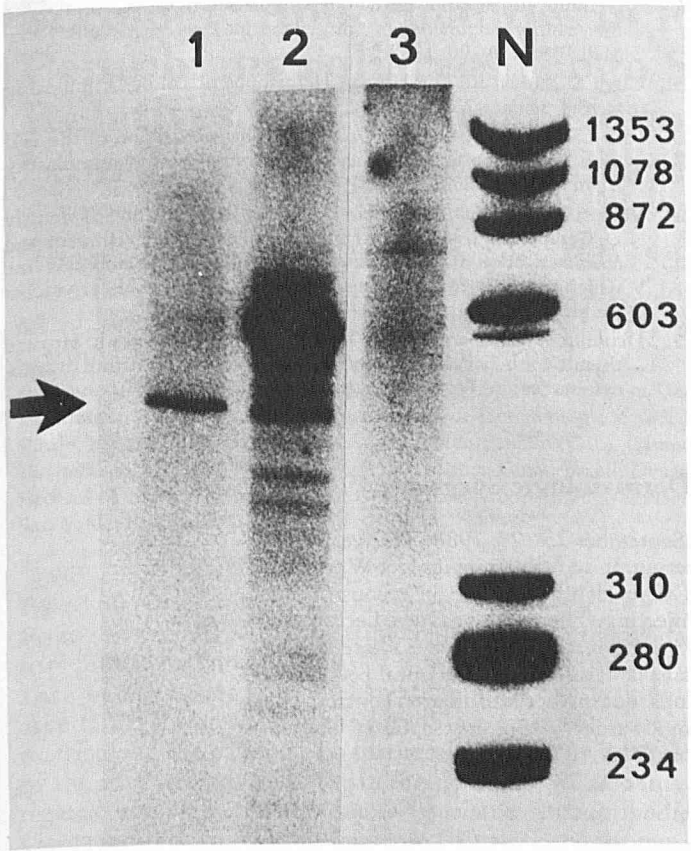

Figure 2. S1 nuclease analysis (see *Materials and Methods)* of poly-A+ RNA from unstimulated Pam 212 cells *(lane 1),* total RNA from Cos 7 monkey cells transfected with pCD-mGM-CSF *(lane 2),* and no RNA *(lane 3).* Molecular weight markers are shown in *lane 4.*

by immunoblot analysis of recombinant GM-CSF and Pam 212 conditioned medium using the neutralizing antibody described above is similar.

Taken together, the above observations lend strong support to the likelihood that KTGF and GM-CSF are identical. Thus, as we have previously reported KTGF would appear to be a misnomer, because this molecule induces not only proliferation of certain T cells, but also of myelomonocytic cell lines and bone marrow cells. It was also recently reported that keratinocyte GM-CSF induced DNA synthesis in murine peritoneal macrophages [16] and enhanced antibody responses in mice by improving antigen-presenting cell function [17]. In addition, it was recently reported that GM-CSF is critical for the in vitro maturation of freshly isolated Langerhans cells into highly efficient antigen-presenting cells that resemble splenic dendritic cells functionally and morphologically [18]. The effects of GM-CSF on mature neutrophil function have been extensively studied [19].

However, with the exception of several recent studies GM-CSF has not been reported to possess direct T-cell activating properties. The inability of investigators to demonstrate GM-CSF receptors on normal T cells suggests that under normal in vitro conditions, GM-CSF is not essential for the growth of T cells. It is not known, however, whether GM-CSF receptors are transiently expressed during T-cell activation; because GM-CSF appears to be produced by all classes of T helper cell upon activation [2], this possibility seems worthy of investigation. However, the demonstration of GM-CSF receptors and responsiveness in certain human and murine lymphoma and leukemia cells and cell lines [20,21], in addition to observation that certain population of thymocytes [22] and certain Thy-1+ dendritic epidermal cell lines appear to respond to GM-CSF in vitro (Tigelaar, personal communication), suggest that there are conditions under which GM-CSF could affect the growth and/

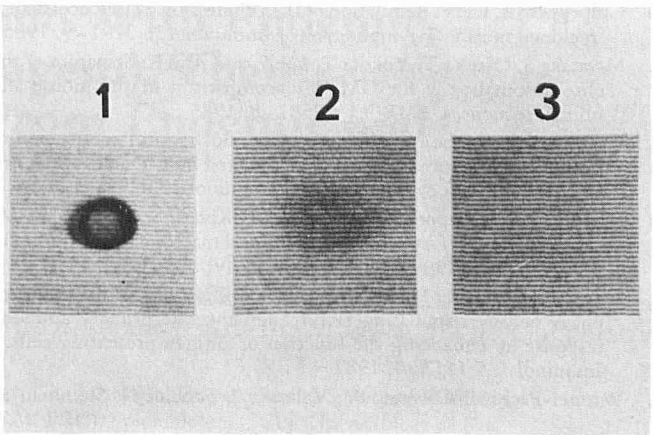

Figure 3. Goat anti-mouse GM-CSF antibody immunoblot analysis of medium containing rGM-CSF *(lane 1),* Pam 212 conditioned medium *(lane 2),* and unconditioned medium *(Lane 3).*

or behavior of T cells, especially in the microenvironment of skin. The availability of recombinant GM-CSF and specific antibodies to this factor will allow for the analysis of a wide range of activities on both mature and precursor T cells.

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