tetramer. This association would result in the presence of 12 to 16 transmembrane regions per synaptophysin homomultimer, a structure very suggestive of a membrane channel. Remarkably, all of the transmembrane regions of synaptophysin are very hydrophobic, with only two containing single charged amino acids. This charge distribution decreases the possibility that synaptophysin is a channel for ions. The hydrophobicity plot and the proposed subunit structure of synaptophysin are very similar to those of the gap junction proteins (*14*).

In summary, the primary structure of synaptophysin suggests that the protein may form a synaptic vesicle–specific membrane channel, with a cytoplasmic domain instrumental in the interactions of synaptic vesicles with cytoplasmic factors.

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2. S. R. Pfeffer and R. B. Kelly, *Cell* **40**, 949 (1985).
3. Since the NH_2-terminus of synaptophysin was blocked, tryptic and chymotryptic peptides, obtained from digests of 100 μg of synaptophysin, were separated by high-performance reversed-phase chromatography on a Vydac 219 TP 300 A phenyl column with a 0 to 70 percent (v/v) acetonitrile gradient in 0.1 percent (v/v) trifluoroacetic acid. One chymotryptic peptide [P1, NIEVEFEYPFR (*4*)] and two tryptic peptides [P2, MATDPENIIKEMP, and P3, APPGAPEKQPAPGD (*4*)] were analyzed by Edman degradation, and the phenylthiohydantoin derivatives were characterized by isocratic high-performance liquid chromatography [F. Lottspeich, *J. Chromatogr.* **326**, 321 (1985)].
4. In the one-letter amino acid code, the letters translate into the following amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.
5. The oligonucleotide was end-labeled (*16*) and used to screen 2×10^6 plaques of a rat brain cDNA library (RL1002, Clontech Lab. Inc.) and 1.5×10^6 plaques of a human retina cDNA library [J. Nathans, C. Thomas, D. S. Hogness, *Science* **232**, 193 (1986)] by standard procedures. After plaque purification, we obtained positive clones and subcloned them into pBR322, pGem 4-1 and M13 mp18 and mp19 vectors, and we analyzed them by the dideoxy sequencing method (*7*). All sequences were determined on both strands.
6. RNA blot analysis was performed (*17*) with single-stranded uniformly labeled DNA probes and with total RNA purified from brains obtained from adult male Wistar rats. The RNA size was determined by comparison with ribosomal RNA standards (Pharmacia) and RNA ladder standards (BRL).
7. T. C. Südhof, F. Lottspeich, P. Greengard, E. Mehl, R. Jahn, *Nucleic Acids Res.*, in press; this report contains the nucleotide and amino acid sequences of rat and human synaptophysin (the sequence will be supplied on request prior to publication).
8. Genomic Southern blots of rat DNA indicated the presence of a single synaptophysin gene. Plaques (2×10^5) of a rat genomic partial Eco RI library (RL1005a Clontech) were screened (*17*) with single-stranded ^{32}P-labeled probes corresponding to nucleotides 116 to 232 of the rat synaptophysin cDNA (*7*). Three identical hybridization-positive clones were isolated. A 3.8-kb Eco RI insert fragment from these was subcloned and analyzed by restriction enzyme mapping and sequencing. This analysis revealed an intron directly 5′ to the beginning of our cDNA clones. To localize the upstream exon, total RNA from rat brain was sequenced directly with an end-labeled oligonucleotide primer complementary to nucleotides 79 to 99 of the cDNA (*7*). RNA sequencing reactions were performed under the conditions of primer extension reactions (*16*), except that the deoxynucleoside triphosphates in the extension solution were exchanged for the appropriate dideoxy mixes. The reaction products were analyzed on sequencing gels, and the sequence was used to design an oligonucleotide to identify the exon in the genomic clone. The sequence of this exon confirmed and extended the RNA sequence. Primer extension analysis suggested a transcription start site 49 bases 5′ to the beginning of our clones. However, S1 nuclease mapping (*17*) with total rat brain RNA and end-labeled single-stranded probes, showed that the 5′ end of the primer extension product artifactually lies in the middle of a contiguous exon and does not represent the transcription start site.
9. Nucleotide sequence data banks (GenBank release 46; EMBL release 9.0) and protein sequence data banks (PSEQIP and NBRF, release 11) were searched for homologous sequences with standard programs. The cytoplasmic domain of synaptophysin was homologous to most glycine- or proline-rich proteins in the data banks simply because of amino acid frequencies. In addition, limited sequence homology was observed between the synaptophysin transmembrane regions and transmembrane regions of cytochrome c from several species. The biological significance of the latter observation remains to be established.
10. The characterized monoclonal antibodies to synaptophysin recognize a cytoplasmic epitope that is collagenase-sensitive, implicating the cytoplasmic tail with its high content of Gly-Pro bonds.
11. The molecular size of rat synaptophysin calculated from the deduced protein sequence is 33,404 daltons, while the experimentally determined value for the monomer of deglycosylated synaptophysin is 34,000 daltons (*1*). The amino acid composition of purified rat synaptophysin was analyzed after vapor-phase hydrolysis at 145°C for 1 hour in 6*N* HCl, 0.1 percent phenol by either the ninhydrin method (method A) or precolumn derivatization with *o*-phthalaldehyde (method B). The following values (molar percent) were obtained (amino acid [method A, method B; nucleotide sequence]): Asx [9.8, 9.4; 7.8]; Glx [9.3, 12.2; 10.5]; Ser [5.5, 6.5; 5.9]; Gly [13.1, 15.0; 12.4]; Thr [4.6, 5.4; 4.9]; His [0.6, 0.9; 0.3]; Ala [10.6, 10.3; 8.8]; Arg [3.1, 3.5; 2.3]; Tyr [5.6, 5.1; 5.2]; Val [7.5, 8.7; 8.1]; Met [1.6, 3.4; 3.3]; Ile [2.9, 2.6; 2.0]; Phe [7.4, 7.6; 7.8]; Leu [8.9, 9.3; 6.5]; Lys [4.3, 5.5; 4.2]; Pro [5.4, -; 6.5].
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Lymphotoxin Is an Important T Cell–Derived Growth Factor for Human B Cells

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Two different assays for B cell growth factors (BCGF) and an antibody against lymphotoxin were used to show that the presence of lymphotoxin in conditioned media derived from normal activated T cells and in a partially purified BCGF accounts for a substantial portion of their B cell growth-promoting activity. A competitive binding assay confirmed the presence of significant amounts of lymphotoxin in the partially purified BCGF. Recombinant lymphotoxin enhanced the proliferation of activated B cells and augmented B cell proliferation and immunoglobulin secretion induced by interleukin-2.

THE ROLE OF VARIOUS SOLUBLE FACtors in the regulation of human B lymphocyte proliferation is somewhat controversial. When activated in vitro with the polyclonal B cell mitogen *Staphylococcus aureus* Cowan I (SAC), human B lymphocytes undergo an initial round of proliferation (*1*); however, this proliferation is short-lived unless exogenous growth factors are added to the cultures. On the basis of this observation an assay was developed for the detection of human B cell growth factors (BCGF) (*2*). Nevertheless the nature of many of the factors that enhance B cell proliferation in this assay remain uncharacterized. Interleukin-2 (IL-2) is one of the growth factors contained in the supernatants conditioned by phytohemagglutinin (PHA)-activated T cells. Although IL-2 enhances the proliferation of SAC-activated B cells (*3*), an antibody to the IL-2 receptor inhibited only 20% to 30% of the BCGF activity present in a T cell supernatant conditioned by PHA-activated T cells (*4*). Recently, we showed that the monocyte product tumor necrosis factor-α (TNF-α), has

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significant BCGF activity in this assay (*5*). Since lymphotoxin is structurally and functionally related to TNF-α and is a product of activated lymphocytes (*6*), we tested the role of lymphotoxin as a growth factor in bulk T cell supernatants. Supernatants conditioned by PHA-activated tonsillar or peripheral blood T cells were incubated with SAC-activated B cells in the presence or absence of sufficient polyclonal antibody to lymphotoxin to neutralize 120 ng of lymphotoxin per milliliter. The presence of the antibody inhibited the amount of DNA synthesis triggered by the PHA-activated T cell supernatants by 71 + 6% (Table 1). The antibody did not inhibit B cell proliferation induced by IL-2, and the inhibitory effects of the antibody could be reversed by adding sufficient exogenous lymphotoxin (400 ng/cm³) (Table 1).

We next tested the effects of the antibody on the proliferation triggered by a commercially available BCGF that had been partially purified from supernatants conditioned by PHA-activated mononuclear cells. The addition of the BCGF, to SAC-activated B cells resulted in a marked increase in B cell DNA synthesis; however, again approximately 70% of this increase could be blocked by the addition of the antibody to lymphotoxin, an indication that this BCGF preparation contained significant amounts of lymphotoxin (Table 1). We also tested the effects of the antibody to lymphotoxin in another assay for human BCGF, the anti-μ costimulation assay. This assay depends on the ability of anti-μ (a rabbit polyclonal antibody directed against the human μ chain coupled to Sepharose) to activate B cells without inducing B cell proliferation unless a source of BCGF is present (*7*). Synthesis of B cell DNA induced with anti-μ and the partially purified BCGF was inhibited (65 ± 8%) by the addition of the antibody. Again, the full proliferative response could be restored by adding an excess of lymphotoxin (Table 1). To further confirm the specificity of the antibody to lymphotoxin we examined its effect on the costimulation of B cells by anti-μ and recombinant IL-4, another factor with BCGF activity (*8*). In contrast to the results with the partially purified BCGF, the antibody did not significantly impair the IL-4 response (Table 1).

Table 1. Inhibition of growth factor–induced B cell proliferation by an antibody to lymphotoxin. Human tonsillar B lymphocytes were purified as described (*7*). More than 95% were surface Ig positive and less than 1% were esterase positive. In experiments 1 and 2, the purified B cells were activated with a 1:12,500 dilution of SAC (Bethesda Research Laboratories) for 2 days in RMPI 1640 containing 10% fetal bovine serum (Dutchland Laboratories). The cells were washed and cultured for 3 or 4 days in Ventrex HL-1 serum-free medium (Ventrex Laboratories) at a concentration of 5×10^5 cells per milliliter in 96-well flat-bottom plates in the presence or absence of various cytokines and in the presence of a polyclonal rabbit antibody to lymphotoxin (sufficient to neutralize 120 ng/ml) (Genentech). PHA supernatants were obtained by culturing tonsillar T cells (supernatant 1) or peripheral blood mononuclear cells (supernatant 2) in serum-free medium for 4 days in the presence of PHA (1 μg/ml). BCGF (Cellular Products) was used at 20% by volume. Recombinant IL-2 (lot LP2, Cetus) was used at 100 U/ml, and recombinant lymphotoxin (Genentech) was used at 400 ng/ml. DNA synthesis was measured during the last 18 hours of the culture period by incorporation of [^{3}H]thymidine (standard liquid scintillation counting). Background thymidine incorporation in medium alone or in the presence of antibody to lymphotoxin was subtracted from the appropriate samples. The antibody either had no effect or a very mild stimulatory effect on baseline B cell proliferation. In experiment 3, three different B cell preparations were stimulated with antibody to μ chain (15 μg/ml) coupled to beads (Bio-Rad) plus the partially purified BCGF in the presence or absence of the antibody to lymphotoxin. Additionally, one B cell preparation was stimulated with anti-μ plus IL-4 at 1000 U/ml (Genzyme). The amount of DNA synthesis was determined as above during the last 18 hours of a 96-hour culture period. Background thymidine incorporation in medium plus anti-μ alone were subtracted from the appropriate samples.

Addition	DNA synthesis (cpm/10^5 B cells)						
	Supernatant 1	Supernatant 2	IL-2	BCGF	BCGF	BCGF	IL-4
			Experiment 1				
None	5,880	31,341	8,287				
Antibody	1,605	11,832	13,832				
			Experiment 2				
None	40,551	26,724	21,797	91,233			
Antibody	10,399	6,181	27,556	28,099			
Antibody + lymphotoxin	51,774	47,376					
			Experiment 3				
None				3,408	26,287	22,232	7,350
Antibody				908	9,404	9,639	6,324
Antibody + lymphotoxin				4,327	20,964	34,784	

To confirm the presence of lymphotoxin in the partially purified BCGF preparation, we used ^{125}I-labeled TNF-α in a competitive binding assay. Since both TNF-α and lymphotoxin bind to the same receptor (*9*), lymphotoxin can be detected by its ability to competitively inhibit the binding of TNF-α (Table 2). As predicted, the commercial BCGF contained significant amounts of lymphotoxin (estimated from a standard curve with recombinant lymphotoxin to be approximately 100 ng/cm³).

We showed that lymphotoxin by itself could enhance B cell DNA synthesis by culturing SAC-activated B cells in the presence of increasing concentrations of recombinant lymphotoxin. There was a dose-dependent enhancement and a 5.3 ± 0.5 increase in DNA synthesis in B lymphocytes stimulated with lymphotoxin at 40 ng/ml compared to cells cultured in media alone (Table 3). The levels of DNA synthesis with an optimal concentration of lymphotoxin were comparable to those achieved with IL-2 at 100 U/ml, a concentration previously shown to be optimal for SAC-activated B cell proliferation (*3*). The addition of lymphotoxin to IL-2 resulted in a further enhancement in IL-2–induced B cell DNA synthesis (Table 3). The combination of IL-2 at 100 U/ml and lymphotoxin at 40 ng/ml resulted in a 14.4 ± 3.7–fold increase in DNA synthesis compared to media alone.

Finally, the effects of lymphotoxin or lym-

Table 2. Competitive inhibition of ^{125}I-labeled TNF-α binding to activated B cells. Purified recombinant TNF-α (Cetus) labeled with Iodobeads (Pierce Chemical Co; specific activity, 5.7 mCi/mg) was biologically active when assayed on L929 cells. Two-day SAC-activated B cells (1×10^6 cells) were incubated with 5000 cpm of ^{125}I-labeled TNF-α (150 p*M*) in the presence or absence of various potential inhibitors in duplicate. After 2 hours of binding at 4°C, cell-associated and free ^{125}I-labeled TNF were separated by placing the reaction mixture over a cushion of 84% silicon oil and 16% paraffin oil and centrifuging at 12,000*g* for 1 minute. The percent inhibition was calculated as $[(SB - BI) \times 100]/SB$, where *SB* is specific binding and *BI* is the specific binding in presence of potential inhibitor. Specific binding is the total amount of radioactivity (disintegrations per minute) bound minus the radioactivity bound in the presence of a 500*M* excess of TNF-α. Similar results were found in two separate experiments.

Concentration (n*M*)	Inhibition (%)			Concentration (%, by volume)	Inhibition (%) BCGF
	TNF-α	Lymphotoxin	IL-2		
0.5	67	55	0	0.5	2
5.0	81	83	9	5.0	27
50	97	89	7	50	80

Table 3. Effect of lymphotoxin on human B cell proliferation and differentiation. Human tonsillar B cells were purified as described in Table 1, but in addition they were depleted of residual monocytes by passage over a G10 column. Two-day SAC-activated B cells were cultured in serum-free media in the presence of various concentrations of lymphotoxin and in the presence or absence of IL-2 (100 U/ml). [^{3}H]thymidine incorporation was measured 4 days later and supernatant Ig production 5 days later with an isotype-specific enzyme-linked immunosorbent assay. Data represent means ± SEM of two experiments. Lymphotoxin had no effect on Ig synthesis in the absence of IL-2. Baseline IgM production was 144 ± 95 ng/ml, and baseline IgG was 95 ± 68 ng/ml.

Lymphotoxin (ng/ml)	DNA synthesis (cpm/10^3 B cells)		IgG (ng/ml)	IgM (ng/ml)
	−IL-2	+IL-2	+IL-2	+IL-2
0	4,944 ± 1,250	29,578 ± 5,983	1,115 ± 638	1,729 ± 570
0.04	7,087 ± 2,113	28,643 ± 7,245	1,347 ± 779	1,845 ± 611
0.4	8,012 ± 1,703	35,374 ± 2,285	1,181 ± 805	2,045 ± 872
4	14,390 ± 2,500	46,483 ± 1,963	1,633 ± 905	2,248 ± 906
40	25,648 ± 4,141	67,185 ± 100	1,529 ± 700	3,746 ± 381
400	29,380 ± 6,591	74,919 ± 2,383	1,426 ± 650	3,330 ± 1,003

photoxin plus IL-2 on B cell immunoglobulin (Ig) secretion were determined. Although lymphotoxin alone had no effect on B cell Ig secretion, the addition of lymphotoxin and IL-2 to SAC-activated B cells resulted in a 1.5- to 2-fold increase compared to IL-2 alone (Table 3). Since B cell Ig production in this system is dependent on antecedent B cell proliferation, lymphotoxin's enhancement of Ig secretion may be secondary to its effects on B cell proliferation.

TNF-α and lymphotoxin are generally defined by their cytotoxic or cytostatic activity against a variety of transformed cells (*10*). Recently Sugarman *et al.* (*11*) have reported that growth of certain untransformed cell lines can be enhanced by TNF-α and Vilcek *et al.* (*12*) noted that TNF-α stimulated the growth of human FS-4 fibroblasts. These bifunctional effects of TNF-α and lymphotoxin on cell growth are not without precedent, since several other growth factors, including IL-2, epidermal growth factor, and transforming growth factor-β, have been reported to either enhance or inhibit cell growth, depending on the particular cell type examined and other growth factors present (*13*).

In conclusion, lymphotoxin is a soluble factor secreted by activated lymphocytes and is present in supernatants conditioned by activated T cells in amounts sufficient to significantly enhance in vitro B cell proliferation. Lymphotoxin may serve an important role in amplifying the humoral immune response in vivo.

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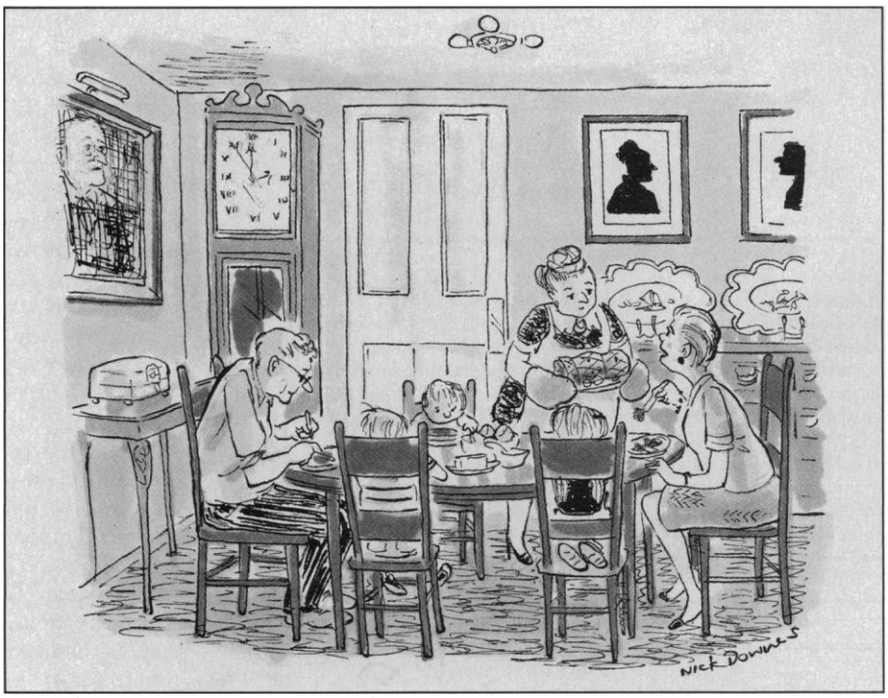

Everything tastes so fresh, Mother. Where did you get the potassium benzoate?