Comets, L. L. Wilkening, Ed. (Univ. of Arizona Press, Tucson, 1982), pp. 383–409.
4. P. Fraundorf, R. I. Patel, S. A. Sanford, R. M. Walker, *Lunar Planet. Sci.* **14**, 207 (1983).
5. Specimens were mounted on carbon support films, either by dispersion onto those films in silicone oil followed by cleaning in hexanes [J. P. Bradley and D. E. Brownlee, in *Microbeam Analysis 1983*, R. Gooley, Ed. (San Francisco Press, San Francisco, 1983), pp. 187–190], or by evaporation of carbon films onto dispersed particles [G. J. Flynn, P. Fraundorf, J. Shirck, R. M. Walker, *Proc. Lunar Planet. Sci. Conf.* **9**, 1187 (1978)]. Care was taken to ensure that observed carbonaceous features were associated with the micrometeorites and not with the support film. Similar features were in fact present in particles mounted by the two distinctly different techniques.
6. P. Fraundorf and J. Shirck, *Proc. Lunar Planet. Sci. Conf.* **10**, 951 (1979); D. E. Brownlee, D. Tomandl, M. B. Blanchard, G. V. Ferry, F. T. Kyte, *NASA Tech. Memo. TMX-73* (1976), p. 152.
7. Structural features of the carbon were investigated by high-resolution lattice fringe imaging. Unlike larger carbonaceous chondrites, most of the carbon in micrometeorites is devoid of observable structure. Occasionally, poorly crystalline carbon is encountered that exhibits turbostratic structure and ~ 3.4-Å (002) interplanar spacings characteristic of graphitic carbon (Fig. 1B).
8. P. B. Hirsch, A. Howie, R. B. Nicholson, D. W. Pashley, M. J. Whelan, *Electron Microscopy of Thin Crystals* (Butterworth, London, 1965), pp. 319–327; S. Nagakura, *J. Phys. Soc. Jpn.* **14**, 186 (1959).
9. L. J. E. Hofer, in *Catalysis*, vol. 4, *Hydrocarbon Synthesis, Hydrogenation, and Cyclization*, P. H. Emmett, Ed. (Reinhold, New York, 1956), pp. 373–441.
10. R. Christoffersen and P. R. Buseck, *Science* **222**, 1327 (1983).
11. P. Fraundorf, *Geochim. Cosmochim. Acta* **45**, 915 (1981).
12. R. T. K. Baker and P. S. Harris, *Chem. Phys. Carbon* **14**, 83 (1978).
13. R. B. Anderson, in *Catalysis*, vol. 4, *Hydrocarbon Synthesis, Hydrogenation, and Cyclization*, P. H. Emmett, Ed. (Reinhold, New York, 1956), pp. 1–371; E. E. Unmuth, L. H. Schwartz, J. B. Butt, *J. Catal.* **63**, 404 (1980); G. D. Renshaw, C. Roscoe, P. L. Walker, Jr., *ibid.* **18**, 164 (1970).
14. R. Hayatsu and E. Anders, *Top. Curr. Chem.* **99**, 1 (1981).
15. J. F. Kerridge, *Earth Planet. Sci. Lett.* **64**, 186 (1983); R. H. Becker and S. Epstein, *Geochim. Cosmochim. Acta* **46**, 81 (1982).
16. J. M. Greenburg, paper presented at the International Conference on Cometary Exploration, Budapest, Hungary, November 1982.
17. It is possible that some of the carbonaceous structures reflect effects of the brief (< 5-second) thermal pulse suffered by all micrometeorites on atmospheric entry. But the presence of these structures in particles exhibiting otherwise diverse, unequilibrated textures (*11*) argues against atmospheric entry as a sole explanation. In fact, some of the carbonaceous structures (for example, mantles and rims) appear likely to predate aggregation of the grains into micrometeorites.
18. J. P. Bradley, D. E. Brownlee, D. R. Veblen, *Nature (London)* **301**, 473 (1983).
19. E. Zinner, K. D. McKeegan, R. M. Walker, *ibid.* **305**, 119 (1983).
20. This research was supported by NASA grant NSG 9052. Transmission electron microscopy was performed at the High-Resolution Electron Microscopy Facility, Arizona State University (NSF grant CHE79-16098). We thank R. M. Walker and P. Self for discussions and M. Wheelock and L. Fortson for assistance.

9 August 1983; accepted 1 November 1983

Inhibition of Human Acute Lymphoblastic Leukemia Cells by Immunotoxins: Potentiation by Chloroquine

Abstract. *Immunotoxins containing pokeweed antiviral protein and monoclonal antibodies against human T cells or human transferrin receptor efficiently killed acute lymphoblastic leukemia cells. Chloroquine specifically enhanced the rate of protein synthesis inhibition by immunotoxin. Depending on its concentration, chloroquine (10 to 100 micromolar) reduced by up to 65-fold the amount of immunotoxin required to inhibit protein synthesis in the target cells 50 percent.*

The specificity with which chemotherapeutic drugs act is far from absolute; because of this, side effects may be unacceptably severe. The ability of monoclonal antibodies to seek out specific cell types (*1*) and to image tumors (*2*) in vivo has been demonstrated in several model systems. On the basis of the antigens carried by the target cell, the use of monoclonal antibodies to accurately deliver lethal agents promises to create a new generation of therapies in which side effects may be eliminated or greatly reduced. Passive serum therapy has had only limited success (*3*), but monoclonal antibodies alone may have some role in therapy (*3, 4*). We believe that antibody-drug conjugates (*5*) and immunotoxins [plant toxins or hemitoxins (*6–8*) coupled to monoclonal antibodies (*9*)] will be even more useful and may allow the absolute amount of a highly toxic agent given to a patient to be safely increased. In the present study we used two different immunotoxins containing pokeweed antiviral protein (PAP) directed against antigens found on human acute lymphoblastic leukemia (ALL) T-cell lines and found that chloroquine stimulated the potent inhibitory effect of the immunotoxins.

We previously found that PAP-containing immunotoxins targeted against Thy 1.1 antigen are as effective as ricin A chain–containing immunotoxins at inhibiting protein synthesis in target cells (*10*). Both are active at very low concentrations (nanograms of immunotoxin per milliliter) and show nearly absolute specificity toward Thy 1.1–bearing cells compared to a leukemia cell line bearing Thy 1.2 allotype. Thy 1.1 (*3, 11*) and Thy 1.2 (*12*) monoclonal antibodies prevent growth in vivo of Thy 1.1 and 1.2 leukemia cells, respectively; however, removal of the Fc portion of the antibody eliminates the protection afforded by Thy 1.1 antibody (*11*). The divalent $F(ab')_2$ fragment, coupled by a disulfide bond to PAP, prevents the growth of leukemia cells in vivo, showing that the immunotoxin has a tumor-suppressive effect (*11*).

The transport mechanisms for different immunotoxins are not known and may differ considerably. Not all immunotoxins are cytotoxic (*10*), indicating that transport of immunotoxins with various specificities is mediated through different mechanisms that distribute them to different cellular compartments or that simple binding at the cell surface is not sufficient for internalization.

Agents that affect lysosomal *p*H affect the internalization of various ligands. Therefore we explored the effect of chloroquine, an agent that raises lysosomal *p*H (*13*), on the cytotoxic activity of immunotoxins containing monoclonal antibody 5E9-11 (directed against human transferrin receptors) or T3-3A1 (directed against human T cells) linked to PAP by a disulfide bond (*14*). Figure 1 illustrates the effects of these immunotoxins on three different human cell lines. Both immunotoxins inhibited protein synthesis in HSB-2 cells better than in MOLT-3 or CEM cells. With the more resistant cells, a significant portion of the protein synthesis activity could not be inhibited even at high concentrations of immunotoxin. Immunotoxins T3-3A1-PAP and 5E9-11-PAP inhibited protein synthesis in HSB-2 cells by 50 percent at concentrations from 10 to 40 ng/ml (5.6×10^{-11} to $22 \times 10^{-11} M$). Similar concentrations of PAP-containing immunotoxins were previously found to be similarly effective against Thy 1.1 antigen (*10*).

Two control experiments showed the specificity of chloroquine's enhancement of immunotoxin action. First, neither T3-3A1-PAP nor 5E9-11-PAP (4.5 μg/ml) inhibited AKR SL3 cells, a spontaneous T-cell lymphoma line from AKR/J mice. Second, an immunotoxin (2.9 μg/ml) containing 31-E6 monoclonal antibody directed against a mouse T-cell antigen (Thy 1.1) and PAP was unable to inhibit protein synthesis in HSB-2 cells. Immunotoxin 31-E6-PAP does not cross-react with human HSB-2 cells and inhibits 50 percent of protein synthesis in AKR SL3 cells at 190 ng/ml (*10*).

Chloroquine at 100 μ*M* inhibited protein synthesis by 50 to 60 percent under the standard assay conditions (overnight incubation of 1×10^5 cells per 0.2 ml); 50 μ*M* chloroquine usually had no effect on protein synthesis but occasionally inhibited it by 10 to 15 percent. Chloro-

quine at concentrations not affecting overall protein synthesis in control cells promoted the cytotoxicity of 5E9-11-PAP immunotoxins (Fig. 2A). Incubation in 10 μM chloroquine reduced the amount of immunotoxin needed to inhibit 50 percent of protein synthesis to 20 ng/ml from 65 ng/ml ($3.6 \times 10^{-10} M$) in its absence. Increasing the concentration of chloroquine to 50 μM further decreased this value to 9 ng/ml ($5.0 \times 10^{-11} M$). When the concentration of chloroquine was raised to 100 μM there was a loss of protein synthesis activity in the controls, but the amount of immunotoxin needed to achieve 50 percent inhibition of protein synthesis was reduced to nearly 1 ng/ml ($5.6 \times 10^{-12} M$). The potentiation of immunotoxin activity was a specific effect since AKR SL3 cells were equally resistant to high concentrations of immunotoxin in the presence or absence of 50 μM chloroquine. We further demonstrated the specificity of the enhanced activity of specific immunotoxins by showing that 31-E6-PAP (2.9 μg/ml, a 30-fold excess over the concentration of T3-3A1-PAP that completely blocks protein synthesis in HSB-2 cells) still did not inhibit protein synthesis in HSB-2 cells in the presence of 40 μM chloroquine.

The promotional effect of chloroquine was not limited to the immunotoxin directed at human transferrin receptor. Fifty micromolar chloroquine reduced the amount of T3-3A1-PAP needed to achieve 50 percent inhibition of protein synthesis from 20 to 1.5 ng/ml (Fig. 2B). T3-3A1-PAP was more cytotoxic than 5E9-11-PAP.

Antigen 3A1 is present in all three ALL T-cell lines. However, line HSB-2 has relatively higher amounts of the antigen than MOLT-3 or CEM-CCL (*15*). This difference in antigen density could account for the higher sensitivity of HSB-2 cells to the immunotoxins, but MOLT-3 and CEM-CCL were resistant even to very high immunotoxin concentrations. The promotional effect appears to be general and not restricted to the two conjugates described here because another PAP-containing immunotoxin directed against ALL B cells was also potentiated by chloroquine (*16*).

Chloroquine speeds the rate at which protein synthesis is inhibited. Figure 3 shows the kinetics of protein synthesis inhibition by the same three concentrations of 5E9-11-PAP in the absence (Fig. 3A) and presence (Fig. 3B) of 40 μM chloroquine. In the absence of chloroquine 5E9-11-PAP inhibited protein synthesis with a lag period at the lowest concentration of immunotoxin used (43 ng/ml). This is in line with our earlier observations with Thy 1.1 antibodies, but is not in agreement with the results of Youle and Neville (*17*). At higher concentrations, however, no lag period was observed. The time required for 50 percent inhibition of protein synthesis at the highest immunotoxin concentration used was about 3.5 hours and first-order kinetics were observed.

In the presence of chloroquine the lag period observed at low immunotoxin concentrations was abolished and considerably faster inhibition of protein synthesis was achieved. At the two higher immunotoxin concentrations the kinetics of inhibition were biphasic. The highest immunotoxin concentration inhibited protein synthesis 50 percent in about 1 hour, compared to 3.5 hours in the absence of chloroquine. Corresponding times for the intermediate immunotoxin concentration were 2 hours in the presence of chloroquine and 6 hours in its absence. The kinetics of inhibition in the presence of chloroquine and PAP-immunotoxin are about the same as those of ricin–Thy 1.1 antibody conjugates (*18*) and are faster than inhibition by Thy 1.1–ricin immunotoxin in the presence of galactose. A similar stimulation of cytotoxicity with ricin A chain–containing immunotoxins has been observed on cultured cells by 10 mM NH_4Cl, another lysosomotropic agent (*19*).

Chloroquine may stimulate immunotoxin-mediated inhibition of protein synthesis by increasing the amount of immunotoxin internalized, preventing the degradation of the immunotoxin once inside the cell (leading to a faster accumulation of threshold levels of immunotoxin), changing the compartmentalization of the immunotoxin to a more favorable location, or enhancing the release of the immunotoxin from acidic compartments. The chloroquine effect was specific since it did not induce immunotoxin-mediated cytotoxicity in nontarget cells (Fig. 2A). Chloroquine does not inhibit the binding of specific immunoglobulin G (IgG) directed against plasma membrane antigens (*20*). Antigen modulation and internalization are critical elements in the activity of conjugates of common ALL antigen and ricin A chain (*21*). Our preliminary data indicate that chloroquine enhances the amount of immunotoxin bound or internalized by the target cells.

A number of phenomena may affect the efficacy of immunotoxin action in vivo and its ability to reach its target. These include antigen shedding and

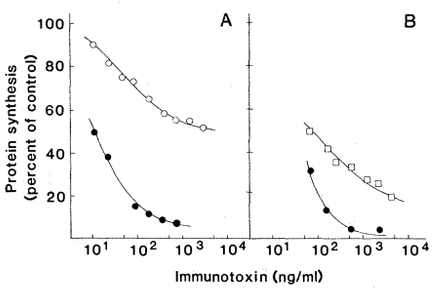

Fig. 1. Effect of PAP-linked immunotoxins on human ALL T-cell lines. Human T-cell lines HSB-2 (●), MOLT-3 (○), and CEM-CCL-119 (□) (*28*) were grown in RPMI 1640 medium supplemented with 20 percent fetal bovine serum (Dutchland Laboratories), 2.0 mM glutamine, and 50 μM β-mercaptoethanol. The cells (1×10^5 in a volume of 180 μl) were mixed with 20 μl of T3-3A1-PAP (A) or 5E9-11-PAP (B). After incubation for 18 hours at 37°C, the cells were centrifuged at 500*g* for 6 minutes and resuspended in serum-free RPMI 1640 containing [^{3}H]leucine as before (*29*). After a 1-hour pulse exposure the cells were harvested on Gelmen glass fiber filter, washed with saline, precipitated with 5 percent trichloroacetic acid (TCA), and washed with cold ethanol. The radioactivity present in the TCA-insoluble fraction was then determined. Each value is a mean of triplicate cultures.

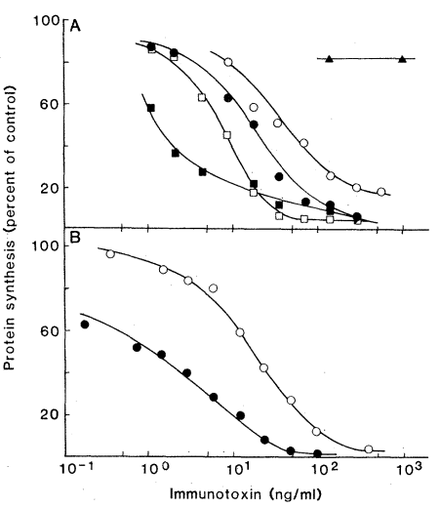

Fig. 2. Dose-dependent enhancement of immunotoxin activity by chloroquine. (A) HSB-2 cells (1×10^5) in a volume of 160 μl were mixed with 20 μl of 5E9-11-PAP and 20 μl of chloroquine or medium to make a final concentration of 10 μM (●), 50 μM (□), 100 μM (■), or 0 μM (○) chloroquine. After incubation for 18 hours under standard culture conditions, the extent of protein synthesis was determined. As a control, the effect of immunotoxin and chloroquine (50 μM) was checked on nontarget AKR SL3 cells (▲). The same amount of protein synthesis was obtained in the presence and absence of chloroquine at both immunotoxin concentrations. (B) Human T-cell antibody linked to PAP (T3-3A1-PAP) was tested for cytotoxicity in the presence (●) or absence (○) of 50 μM chloroquine as described above.

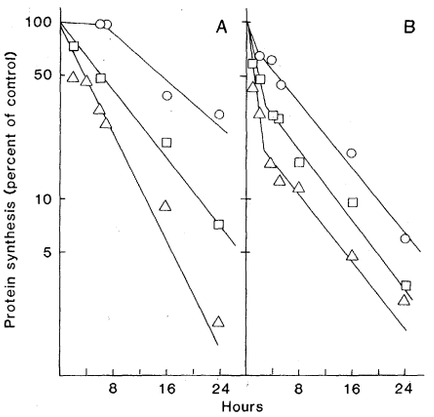

Fig. 3. Kinetic changes in immunotoxin action induced by chloroquine. The toxicity of 43 ng (○), 430 ng (□), and 4.3 μg (△) of 5E9-11-PAP immunotoxin per milliliter toward HSB-2 cells was determined after the indicated incubation periods in the absence (A) or presence (B) of 40 μ*M* chloroquine.

modulation, circulating antigens, tumor vascularization, tumor heterogeneity and absence of antibody receptors on some tumor cells, immunogenicity of the immunotoxin, and rate of clearance and degradation of immunotoxin. Nevertheless, some successes have been reported in treating tumor-bearing (*22*) or tumor-challenged mice (*11, 12*) with immunotoxins, and there have been several instances in which tumor cells in culture were specifically killed by immunotoxins (*10, 12, 21, 23, 24*). Immunotoxins may be of considerable value in eliminating metastatic cells after surgery. Their use outside the body in bone marrow transplantation has even more potential.

The use of PAP-containing immunotoxins in vivo offers several advantages over ricin A chain–containing immunotoxins. The lack of an analog of ricin B chain in PAP circumvents the difficult task of purifying ricin A chain, greatly reducing problems of nonspecificity encountered because of contaminating ricin B chain in preparations of ricin A chain used for conjugation to antibodies. The effectiveness of disulfide-linked immunotoxins will be eliminated if reduction of the disulfide bond occurs in vivo. One particularly important advantage is the retention of cytotoxicity when PAP and antibody are coupled by a noncleavable cross-link (*10*), in contrast to the loss of cytotoxicity when ricin A chain and antibody are similarly linked (*25*). Because the three species of PAP are immunologically distinct and at least two of them are actually different proteins (*8*), the PAP species in the immunotoxin can be easily switched and their activity retained if antibodies against the immunotoxins are raised in an immunotoxin-treated patient.

If the rate of tumor cell killing by immunotoxins is slow in relation to the rate of tumor cell proliferation, then therapy will not succeed. For this reason, and because chloroquine has long been used safely at high doses (0.3 g/day) for long times, the use of chloroquine treatment in conjunction with immunotoxins may be useful in vivo. However, its more immediate application may be in treating bone marrow in vitro. The rate of killing of cultured cells by ricin A chain–containing immunotoxins has generally been slow compared to the rate of killing by ricin. Depending on the dose, ricin can completely inhibit protein synthesis in 30 to 40 minutes, but ricin A chain–containing immunotoxins generally require 6 to 24 hours to inhibit protein synthesis by 50 percent. Ricin-containing immunotoxins are less specific due to the B-chain carbohydrate binding site in ricin, but addition of galactose or lactose can partially prevent this nonspecificity. The carbohydrate binding site on ricin-containing immunotoxins may also be blocked by a photoactivable derivative of galactose (*26*).

Much attention is being focused on immunotoxin treatment for autologous or heterologous bone marrow transplantation. Immunotoxins seem particularly well suited for this since treatment of the cells can be done outside the body. The kinetics of killing tumor cells and T cells by immunotoxins are important because exposing bone marrow preparations for long periods at room temperature can significantly reduce the viability of stem cells. This presents particular problems for complement-mediated killing of tumor cells. Moreover, such killing requires much higher concentrations of antibody than of an immunotoxin to eliminate BCL_1 cells in bone marrow preparations (*24*). Chloroquine could enhance the kinetics of immunotoxin action or reduce the amount of immunotoxin required, resulting in better preservation of the stem cell populations. Because T3-3A1-PAP is so potent in killing human T-cell lines and because all ALL T-cell lines tested have been positive for 3A1 (*27*), it may be possible to use this immunotoxin to remove ALL T cells and mature T cells for autologous and allogeneic bone marrow transplantation.

S. RAMAKRISHNAN
L. L. HOUSTON
Department of Biochemistry, University of Kansas, Lawrence 66045

References and Notes

1. L. L. Houston, R. C. Nowinski, I. D. Bernstein, *J. Immunol.* **125**, 837 (1980).
2. B. Ballou, G. Levine, T. R. Hakala, D. Solter, *Science* **206**, 844 (1979); D. M. Goldenberg, E. E. Kim, F. H. DeLand, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7754 (1981); D. Herlyn *et al.*, *Cancer Res.* **43**, 2731 (1983); D. Colcher *et al.*, *ibid.*, p. 736.
3. P. W. Wright and I. D. Bernstein, *Prog. Exp. Tumor Res.* **25**, 110 (1980); M. E. Kirch, T. Lee, U. Hämmerling, *Res. Monogr. Immunol.* **3**, 221 (1981).
4. S. J. Kennel, T. Lankford, K. M. Flynn, *Cancer Res.* **43**, 2843 (1983); C. C. Badger and I. D. Bernstein, *J. Exp. Med.* **157**, 828 (1983); R. A. Miller, M. G. Maloney, J. McKillop, R. Levy, *Blood* **58**, 78 (1981).
5. Y. Tsukada *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 621 (1982); M.-T. B. Davis and J. F. Preston, III, *Science* **213**, 1385 (1981).
6. Ricin, a plant toxin, has an A chain (ribosome-inhibiting) and a B chain (cell surface–binding). Pokeweed synthesizes polypeptides similar to ricin A chain in function and size (*7*); since it has no B chain to facilitate delivery, we refer to it (and other similar proteins) as a hemitoxin. When the pokeweed protein is coupled to ricin B chain, it becomes cytotoxic (*8*). At least three species of pokeweed antiviral protein exist: PAP from spring leaves, PAP-II from late summer leaves, and PAP-S from seeds. All three have molecular weights of about 30,000 and do not cross-react with specific antibodies. Although they have about the same ability to inactivate ribosomes, PAP and PAP-S are nonidentical proteins differing in 19 of the 32 amino terminal residues (*8*).
7. L. Barbieri and F. Stirpe, *Cancer Surv.* **1**, 489 (1982); J. D. Irvin, *Pharmacol. Ther.*, in press.
8. L. L. Houston, S. Ramakrishnan, M. A. Hermodson, *J. Biol. Chem.* **258**, 9601 (1983).
9. S. Olsnes and A. Pihl, *Pharmacol. Ther.* **15**, 355 (1982); E. S. Vitetta, K. A. Krolick, M. Miyama-Inaba, W. Cushley, J. W. Uhr, *Science* **219**, 644 (1983).
10. S. Ramakrishnan and L. L. Houston, *Cancer Res.*, in press.
11. ———, in preparation.
12. H. E. Blythman *et al.*, *Nature (London)* **290**, 145 (1981).
13. D. J. Reijngoud and J. M. Tager, *Biochim. Biophys. Acta* **472**, 419 (1977); S. Ohkuma and B. Poole, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3327 (1978).
14. PAP (spring leaf) was purified by carboxymethyl cellulose chromatography (*8*). Cells for monoclonal antibody production were obtained from the American Type Culture Collection (ATCC). Ascites fluid was collected from mice and the antibodies were isolated by sodium sulfate precipitation and diethylaminoethyl cellulose (DEAE–Bio-Gel) or Affi-Gel Blue chromatography. The antibodies were linked to PAP with *N*-succinimidyl-3-(2-pyridyldithio)propionate (*10, 18*). The immunotoxins were isolated by gel filtration and analyzed by radioimmunoassay [S. Ramakrishnan, M. E. Eagle, L. L. Houston, *Biochim. Biophys. Acta* **719**, 341 (1982)] and sodium dodecyl sulfate–gel electrophoresis. For calculations of molar concentrations we used a molecular weight of 180,000 for all immunotoxins. Monoclonal antibodies produced in mice were T3-3A1 (ATCC No. HB-2), an IgG1 (κ) antibody to human T cell that binds to 65 percent of human peripheral blood thymus-derived cells and is obtained by immunizing mice with HSB-2 human T cells [B. F. Haynes, G. S. Eisenbarth, A. S. Fauci, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5829 (1979)] and 5E9-11 (ATCC No. HB-21), an IgG1 (κ) antibody to human transferrin receptor derived from mice immunized with HSB-2 human T cells [B. F. Haynes *et al.*, *J. Immunol.* **127**, 347 (1981)].
15. B. F. Haynes, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2914 (1980).
16. F. M. Uckun, S. Ramakrishnan, L. L. Houston, unpublished data.
17. R. J. Youle and D. M. Neville, Jr., *J. Biol. Chem.* **257**, 1598 (1982).
18. L. L. Houston and R. C. Nowinski, *Cancer Res.* **41**, 3913 (1981).
19. P. Casellas, H. E. Blythman, P. Gros, B. Richer, F. K. Jansen, *Protides Biol. Fluids Proc. Colloq.* **30**, 359 (1983).
20. Y.-J. Schneider and A. Trout, *Eur. J. Biochem.* **118**, 33 (1981).
21. V. Raso, J. Ritz, M. Basala, S. F. Schlossman, *Cancer Res.* **42**, 457 (1982).
22. K. A. Krolick, J. W. Uhr, S. Slavin, E. S. Vitetta, *J. Exp. Med.* **155**, 1797 (1982).

23. R. J. Youle and D. M. Neville, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5483 (1980); T. N. Oeltman and J. T. Forbes, *Arch. Biochem. Biophys.* **209**, 362 (1981); L. L. Houston and R. C. Nowinski, *Cancer Res.* **41**, 3913 (1981); I. S. Trowbridge and D. L. Domingo, *Nature (London)* **294**, 171 (1981).
24. K. A. Krolick, J. W. Uhr, E. S. Vitetta, *Nature (London)* **295**, 604 (1982).
25. Y. Masuho *et al.*, *J. Biochem. (Tokyo)* **91**, 1583 (1982).
26. L. L. Houston, *J. Biol. Chem.* **258**, 7208 (1983).
27. I. Royston *et al.*, *Proc. Am. Soc. Clin. Oncol.* **2**, c-690 (1983).
28. Cell lines (all derived from peripheral blood of ALL patients) were MOLT-3 (ATCC No. CRL 1552) [J. Minowada, T. Ohnuma, G. E. Moore, *J. Natl. Cancer Inst.* **49**, 891 (1972)], CCRF-CEM (ATCC No. CCL 119) [*Cancer Res.* **27**, 772 (1967)], and CCRF-HSB-2 (ATCC No. CCL 120.1) [*ibid.* **28**, 1121 (1968)]. AKR SL3 is a spontaneous lymphoma derived from AKR/J mice.
29. L. L. Houston, *J. Biol. Chem.* **257**, 1532 (1982).
30. Supported by grant CA 29889 from the National Cancer Institute.

2 September 1983; accepted 3 November 1983

Coexistence of Acetylcholinesterase and Somatostatin-Immunoreactivity in Neurons Cultured from Rat Cerebrum

Abstract. *Cultures derived from rat cerebral hemispheres were sequentially stained for acetylcholinesterase activity and for either somatostatin-like immunoreactivity or cholecystokinin-like immunoreactivity. Somatostatin-like immunoreactivity was found to coexist with acetylcholinesterase activity in individual neurons of several morphological subtypes, but cholecystokinin-like immunoreactivity and acetycholinesterase activity were never seen in the same neurons. These findings suggest a specific anatomical association, perhaps even an overlap, of the cholinergic and somatostatinergic systems in the mammalian cerebrum, and indicate that the combined deficiencies of somatostatin and cholinergic markers in Alzheimer's dementia and senile dementia of the Alzheimer type may be of pathophysiological importance.*

Numerous studies have demonstrated that there are relatively selective and profound decreases in cholinergic markers such as choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in cerebral cortex and hippocampal formation in Alzheimer's disease and senile dementia of the Alzheimer type (AD/SDAT) (*1–4*). More recently, studies in several laboratories have shown that there is also a significant decrease in the assayable levels of somatostatin (SOM) in AD/SDAT (*5, 6*). Levels of six other neuropeptides, including vasoactive intestinal polypeptide (*7, 8*), arginine vasopressin (*9*), cholecystokinin (CCK) (*8*), and thyrotropin-releasing hormone, luteinizing hormone–releasing hormone, and substance P (*10*) have been reported as being unchanged in AD/SDAT, and an additional study reported only small decreases in substance P levels as compared with the larger decreases in SOM (*11*). These data therefore suggest that the decrease in SOM, like that of the cholinergic markers, is relatively selective. However, to date there has been no evidence to suggest possible neuronal associations of the cholinergic and somatostatinergic systems in the brain, and at least three recent studies have suggested that the two systems do not overlap (*12–14*).

Cultures of dispersed cells derived from cerebrum of fetal rats have proved to be useful for analysis of physiological, biochemical, and pharmacological properties of cortical neurons (*15–18*). All of these studies have emphasized the similarities of neurons in culture with those of comparable neurons in situ. Neurons in these cultures have been characterized by numerous techniques, including both silver and tetanus toxoid staining and by extensive intracellular electrophysiological recordings (*15*), and more recently by morphological classification after intracellular dye injections (*19*), such that individual cells can be readily identified as neurons on the basis of their phase-contrast appearance in either the living or fixed state.

Immunohistochemical staining has delineated a neuronal localization of several neuropeptides including SOM (*20*) and cholecystokinin octapeptide (CCK-8) (*21*), and the cultures have subsequently been used to study the neuronal biosynthesis and release of SOM (*22, 23*) and to analyze some of the complexities of the neuronal response to SOM (*24*). A previous study has also demonstrated the feasibility of histochemical staining for AChE in these cultures (*25*). In the present study we utilized a sequential staining technique for AChE activity and then for either SOM-like immunoreactivity (SOM-LI) or CCK-8-like immunoreactivity (CCK-8-LI) to determine whether one or both of these peptides might coexist with AChE in the cultured cerebral neurons. Our findings of the frequent coexistence of SOM-LI and AChE in individual neurons suggest a relationship between the cholinergic and somatostatinergic systems in the mammalian cerebrum.

Dissociated cell cultures were prepared from cerebral hemispheres of 15- to 16-day-old rat embryos (*15, 16*). Cultures were grown on collagen and polylysine-covered glass cover slips in minimum essential medium and 5 percent rat serum in a 5 percent CO_2 atmosphere at 36°C. Cultures were inhibited with cytosine arabinoside for 24 hours when background cells reached confluency. Studies were performed on mature neurons in culture 4 to 8 weeks after plating. To enhance staining for both AChE and SOM, cultures were first incubated with colchicine (100 n*M*) for 24 hours immediately prior to fixation. Cultures were then rinsed in a Hepes buffer (NaCl, 140 m*M*; Hepes, 20 m*M*; glucose, 10 m*M*; $MgSO_4$, 1.5 m*M*; K_2HOP_4, 1.5 m*M*; $CaCl_2$, 1.8m*M*; brought to *p*H 7.4 with tris), fixed at room temperature in 4 percent paraformaldehyde for 30 minutes followed by 5 percent dimethyl sulfoxide for 30 minutes.

AChE histochemistry was performed by a method modified from Koelle (*26*). Cover slips were incubated overnight in a mixture of 4 m*M* acetylthiocholine iodide (Sigma), 10 m*M* glycine, and 2 m*M* copper sulfate in 50 m*M* sodium acetate (*p*H 5.0) at 4°C. Ethopropazine (0.2 m*M*) (*27*) was included in this mixture to inhibit nonspecific cholinesterases. The stain was developed in 40 m*M* sodium sulfide (*p*H 7.8) for 150 seconds followed by an intensification in a freshly prepared 1 percent silver nitrate solution for 2 minutes at room temperature.

For immunohistochemical staining for SOM-LI and CCK-LI, cover slips were first incubated for 1 hour at 36°C under a drop (40 μl) of a 1:200 dilution of a rabbit polyclonal antibody raised against either bovine serum albumin–conjugated cyclic SOM-14 (*28*) or against hemocyanin–*p,p'*-difluoro-*m,m'*-dinitro–diphenyl sulfone–conjugated CCK-8 (anti-CCK-8) (Immunonuclear Corporation). The second incubation was with a 1:30 dilution of an FITC-conjugated goat antiserum to rabbit immunoglobulin (Cappel Laboratories) for 1 hour at 37°C. Rinsing was accomplished using Hepes buffer to which 0.2 percent Triton-X had been added. For the CCK-8 staining only, the cover slips were incubated with nonimmune goat serum 1:1 for 30 minutes prior to each immunoglobulin incubation. Cover slips were mounted in buffered glycerol (*p*H 9.0) and examined using a Zeiss Universal microscope equipped for epifluorescence, bright field, and phase