give a clue to understanding the stimulation of mucus release in the body. Although we did not find mucus-stimulating activity in the culture fluid of the cells—even after heating to 85°C for 4 minutes—MSS might be released from lymphoblastoid cells into the serum in vivo. With a different assay involving the use of isolated rabbit endocervical cells, Nicosia *et al.* (*7*) found an MSS in rabbit serum. Fetal calf serum in the culture medium of the lymphoblastoid cells was not found to contain MSS's, even after heating.

The finding that trypsin and deoxyribonuclease I affects the activity in the nuclear fraction but not in the cytoplasmic fraction suggests the presence of at least two different MSS's in the cell. The effects of the nucleotides seem to indicate the involvement of energy-dependent processes in the activation of MSS's. Although the concentration at which the maximal response was obtained, $10^{-2}M$, is fairly high, it seems to be specific for the nucleoside triphosphates and for the Mg-nucleotide complex. The increase in titer after addition of deoxyribonuclease I may also be due to the endogenous formation of nucleoside triphosphates, since it occurs only with the nuclear fraction. The relation between the two MSS fractions can be determined only after further characterization and purification.

H. KULEMANN-KLOENE
Department of Pathobiology, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205

S. S. KRAG
Department of Biochemistry, Johns Hopkins University School of Hygiene and Public Health

F. B. BANG
Department of Pathobiology, Johns Hopkins University School of Hygiene and Public Health

References and Notes

1. J. Cantacuzène, *Arch. Roum. Pathol. Exp. Microbiol.* **1**, 7 (1928).
2. B. G. Bang and F. B. Bang, *Johns Hopkins Med. J.* **145**, 209 (1979).
3. ———, *Biol. Bull. (Woods Hole, Mass.)* **159**, 571 (1980).
4. Line MGL-8 was obtained from J. W. Littlefield, Department of Pediatrics, Johns Hopkins University School of Medicine.
5. Line GM 0130 was obtained from the Human Genetic Mutant Cell Repository, Camden, N.J.
6. The deoxyribonuclease I (Sigma) used was derived from bovine pancreas. It contained about 2000 Kunitz units per milligram of protein and was substantially free of ribonuclease.
7. S. V. Nicosia, E. J. Streibel, L. Mogul, *In Vitro* **17**, 205 (1981).
8. We thank B. G. Bang for advice on the urn cell assay and for providing urn cells, P. C. Huang for advice on cell homogenization, and J. W. Littlefield for providing MGL-8 cells. Supported by NIH grant 5P50 HL-19157 and NCI grant CA-00640.

21 June 1982

Transformation of Human Leukocytes by Cocultivation with an Adult T Cell Leukemia Virus Producer Cell Line

Abstract. *The transmission of adult T cell leukemia virus, a human retrovirus, into fresh leukocytes from normal humans was examined. One of three virus-carrying cell lines, tested after being subjected to lethal x-irradiation, consistently transformed leukocytes from adult peripheral blood and umbilical cord blood. All the transformed cell lines expressed adult T cell leukemia virus–associated antigen, but transformed lines originating from adult and umbilical cord blood exhibited T cell and non-T, non-B cell surface natures, respectively. Efforts to transform human leukocytes with cell-free virus were unsuccessful.*

In 1977 Takatsuki and his colleagues (*1*) reported the clinical and hematological characters of a new type of leukemia, adult T cell leukemia (ATL). Since then, many investigators have confirmed the existence of this malignancy and have proposed that the disease also be called adult T cell leukemia/lymphoma because of its leukemic, lymphomatous nature (*2*). The disease is principally found in areas of southwestern Japan.

Recently, Hinuma *et al.* (*3*) reported that an antigen associated with a long-term culture of ATL cell line MT-1 reacted with serum from all ATL patients tested and also with serum from about 25 percent of the healthy adults tested in areas where ATL is endemic. The antigen (ATLA) reacted with very few serum samples from subjects living in areas not affected by ATL. This antigen is also found in two other cell lines, MT-2 (*4*) and MT-4 (*5*), each of which was obtained by coculturing peripheral leukemic cells from ATL patients with normal umbilical cord leukocytes. Both MT-2 and MT-4 were shown to be of cord cell origin by sex chromosome analysis. Furthermore, ATLA was detectable in short-term cultures of peripheral leukemic cells from ATL patients (*6*). Thus ATLA was consistently detected in long- or short-term cultures of ATL cells but not in 14 other human lymphoblastoid cell lines, including T cell, B cell, and non-T, non-B cell lines (*3*).

Virus particles with C-type morphology were found in all the ATLA-positive cell cultures by electron microscopy (*3, 4, 6, 7*). We have demonstrated and characterized type C retrovirus by biochemical techniques (*8*) and have named the virus adult T cell leukemia virus (ATLV). However, the biological activities of this retrovirus are largely unknown. We now report the transmission of ATLV genomes into normal human adult peripheral and umbilical cord blood leukocytes by cocultivation with x-irradiated MT-2 cells (*9*). Transmission was revealed by the transformation of and expression of ATLA in the recipient cells.

Cells of line MT-1, MT-2, or MT-3 were cultured in RPMI-1640 medium (supplemented with 20 percent fetal calf serum and antibiotics) at 37°C in an incubator with CO_2. About half the medium was changed twice a week. The cells were lethally x-irradiated (9000 R), centrifuged, and suspended in fresh medium to reduce the concentration of free radicals generated in the medium during irradiation. Blood was obtained from healthy adults and from umbilical cords at full term. Mononuclear leukocytes (lymphocytes and macrophages) were prepared by Ficoll-Conray gradient centrifugation (*10*). Samples of freshly prepared leukocyte suspension (1×10^6 cells per milliliter) or cells that had been cultured for 1 day were mixed with an equal volume of the suspension of irradiated MT-2 cells (1×10^6 cells per milliliter). Samples (1.0 ml) of the mixtures were cultured in plastic wells (Falcon model 3008). Control cultures consisted of leukocytes or irradiated MT-2 cells alone. The cultures were examined at least once a week for cell transformation, which was defined as the appearance of scattered cell aggregations and a subsequent increase in their size and number. Transformation was confirmed by continuous growth of the cells during serial subcultivation.

In cocultures with irradiated MT-2 cells, transformation and continuous growth of "recipient" cells from adults or newborns were observed after 2 to 4 weeks of incubation. In control cultures the irradiated MT-2 cells did not show any evidence of proliferation, and cultures containing only leukocytes showed only transient proliferation of macrophages or lymphoid cells. All 20 of the transformed cell lines tested were positive for ATL virus.

Table 1 shows properties of 12 transformed cell lines derived from six preparations of adult peripheral blood leukocytes (PBL) and six of umbilical cord blood leukocytes (CBL). The lines show variable proportions of ATL antigen–positive cells (from 17 to over 90 percent). All the lines tested were negative

0036-8075/82/0820-0737$01.00/0

Table 1. Analysis of transformed cells derived from leukocytes from peripheral or umbilical cord blood cocultivated with x-ray–irradiated ATLV-carrying MT-2 cells. N.D., not determined.

Source of specimen	Time in coculture	ATLA-positive cells (%)	Cells with markers (%)				
			Leu 1	Leu 2a	Leu 3a	Leu 4	Immunoglobulin A
Peripheral blood cell line							
OKA	5 weeks	> 90	> 80	0*	> 80	> 80	8
IMA	6 weeks	> 90	> 80	0	8	N.D.	13
YAM	6 weeks	23	> 80	8	> 80	> 80	74
KOM	6 weeks	> 90	> 80	0	22	N.D.	20
AZU	6 weeks	14	> 80	25	> 80	> 80	71
YAN	3 weeks	> 90	> 80	14	> 80	54	2
Umbilical cord blood cell line							
NAK	6 weeks	> 90	2	0	0	0	33
AIB	6 weeks	> 90	0	0	0	0	7
FUK	6 weeks	> 90	0	0	0	0	35
KIY	6 weeks	> 90	3	0	0	0	N.D.
KOS	6 weeks	> 90	15	10	35	32	9
OTS	7 weeks	> 90	14	0	0	1	N.D.
MT-2 cell line							
OHT	> 4 years	> 90	> 80	0	0	0	> 80

*Values of 0 include values < 1 percent.

for Epstein-Barr virus–associated nuclear antigens. They were also negative for surface immunoglobulin. All six PBL lines contained many cells possessing the T cell surface markers Leu 1, Leu 3a, and Leu 4. In contrast, most of the CBL lines did not have surface antigens. Although Leu 1–positive cells were present, their fluorescence was weak and the fraction with this antigen was small. Rosette formation was examined with neuraminidase-treated sheep red blood cells. More than 80 percent of both PBL lines tested formed rosettes, while the three CBL lines tested did not form rosettes. These findings suggest that CBL lines are not T cells but rather are non-T, non-B cells. Karyological studies were performed to determine whether these cell lines originated from recipient or MT-2 cells, which are known to possess the male karyotype. Cell line CBL AIB had a diploid karyotype with female sex chromosomes, showing that it was derived from female recipient and not male "donor" MT-2 cells.

To see whether the susceptibility of target leukocytes to transformation by MT-2 cells differs in individual donors, we determined the minimum number of x-irradiated MT-2 cells necessary to induce transformation of a fixed number of recipient cells (5×10^5 per well). As shown in Table 2, the susceptibility to transformation differed considerably in different individuals; for instance, in leukocytes from donors KOY and MUR, only 1×10^2 MT-2 cells caused transformation, whereas 5×10^5 cells were required for transformation of leukocytes from KAN and SAK. The reason for this difference in susceptibility is unknown.

Next, we examined whether ATLV-carrying cell lines MT-1 and MT-4 have a transforming capability similar to that of MT-2. The preparations of MT-1, MT-2, and MT-4 cells contained 3, > 90, and 50 percent ATLA-positive cells, respectively. Recipient leukocytes were obtained from OKA (Table 1). These leukocytes (5×10^5) were cocultured with the same number of cells from one of the three lines after irradiation. No transformation occurred in cocultures with MT-1 or MT-4 cells, although apparent transformation was consistently observed in those with MT-2 cells.

We also attempted to transform human leukocytes with cell-free virus. Various preparations of ATLV were made from MT-1, MT-2, and MT-4 cell lines as follows. Virus was obtained from fluid or cells (2×10^7 per milliliter) cultured for 1 to 7 days at 37°C. Culture fluids, which contained about 3×10^5 infectious units—as determined by an assay of ATLA-inducing activity (*11*)—were used as is or concentrated 100 times (*8*). In all experiments with six CBL and ten PBL preparations from different individuals, no transformation was observed after infection of these cells with 12 different preparations of ATLV.

Table 2. Susceptibility of leukocytes from normal adult peripheral blood and umbilical cord blood to transformation by cocultivation with MT-2 cells.

Source of specimen	Minimum number of MT-2 cells necessary for transformation per well
Peripheral blood leukocytes	
KOY	1×10^2
OCH	5×10^4
TAK	5×10^4
KAN	5×10^5
YAM	$> 1 \times 10^5$
Cord blood leukocytes	
MUR	1×10^2
KOH	5×10^2
SAK	5×10^5

The transformation of the PBL and CBL lines in the cocultivation procedure (*9*) does not seem to be attributable to cell fusion resulting in hybrids of ATLA-positive MT-2 cells and leukocytes, but rather to the introduction of ATLV genomes from the former cells into the latter by cell-to-cell infection. Three findings support this conclusion. (i) A cell line analyzed karyologically was diploid, with female chromosomes not present in MT-2 cells. (ii) Cell surface marker studies revealed that none of the transformed cell lines closely resembled MT-2 cells. (iii) In separate experiments (*12*), we observed the transmission of ATLV genomes into various ATLA-negative human lymphoid cell lines including T, B, and non-T, non-B cells by cocultivation with irradiated MT-2 cells. These lines became ATLA-positive. These data strongly support the idea that virus particles or genomic materials of ATLV from lethally irradiated, ATLA-positive cells are introduced into normal human leukocytes, with the cells then being transformed. It is not known whether virus infection occurs by cell-to-cell infection. It is possible that the free virus infects after its release into the culture medium. However, this possibility seems unlikely, because no cell-transforming activity of cell-free MT-2 virus was detected in several tests.

The transformed PBL and CBL lines occasionally showed decreased growth ability. Their growth could be restored by adding T cell growth factor. Thus, this growth factor is required for the continuous growth of certain transformed ATLA-positive cells.

Coculture with lines MT-4 and MT-1 did not cause transformation of normal leukocytes. One possible reason for this is that the number of infectious cells in these two lines was too small to induce transformation. Another possibility is that lines MT-1 and MT-4 lack the ability to induce transformation. Analysis at the molecular level is required to determine the mechanism by which human leukocytes in coculture with ATLV-carrying MT-2 cells are transformed.

It was recently reported that human T cell leukemia virus (HTLV), isolated from cells from patients with cutaneous

T cell lymphoma/leukemia in the United States, was reactive with serum from Japanese ATL patients (*13, 14*). Furthermore, patients with a form of ATL indistinguishable from Japanese ATL have been found in the West Indies. The serum of these patients is uniformly positive for HTLV antibodies (*15*). These observations indicate that HTLV is either identical to or closely related to ATLV and that the virus has a worldwide distribution.

NAOKI YAMAMOTO
MORIHISA OKADA, YOSHIO KOYANAGI
MARI KANNAGI, YORIO HINUMA
Institute for Virus Research,
Kyoto University, Kyoto 606, Japan

References and Notes

1. K. Takatsuki, T. Uchiyama, K. Sagawa, J. Yodoi, in *Topics in Hematology*, S. Seno, F. Takaku, S. Irino, Eds. (Excerpta Medica, Amsterdam, 1977), p. 73; T. Uchiyama, J. Yodoi, K. Sagawa, K. Takatsuki, H. Uchino, *Blood* **50**, 481 (1977).
2. The T- and B-Cell Malignancy Study Group, *Jpn. J. Clin. Oncol.* **11**, 15 (1981).
3. Y. Hinuma, K. Nagata, M. Hanaoka, M. Nakai, T. Matsumoto, K. Kinoshita, S. Shirakawa, I. Miyoshi, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6476 (1981).
4. I. Miyoshi, I. Kubonishi, S. Yoshimoto, T. Akagi, Y. Ohtsuki, Y. Shiraishi, K. Nagata, Y. Hinuma, *Nature (London)* **294**, 770 (1981).
5. I. Miyoshi, personal communication.
6. Y. Hinuma *et al.*, *Gann* **73**, 341 (1982).
7. H. Tanaka, personal communication; M. Nakai, personal communication.
8. M. Yoshida, I. Miyoshi, Y. Hinuma, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2031 (1982).
9. W. Henle, V. Diehl, G. Kohn, H. zur Hausen, G. Henle, *Science* **157**, 1064 (1967).
10. A. Boyum, *Scand. J. Clin. Lab. Invest. Suppl.* **21**, 77 (1968).
11. The infectivity of ATLV was determined by monitoring the development of ATLA in phytohemagglutinin-stimulated peripheral lymphocytes from healthy adults by means of the immunofluorescence procedure (*3*).
12. N. Yamamoto *et al.*, in preparation.
13. B. Poiesz, F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, R. C. Gallo, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7415 (1980).
14. M. Robert-Guroff, Y. Nakao, K. Notake, Y. Ito, A. Sliski, R. C. Gallo, *Science* **215**, 975 (1982).
15. D. Catovsky *et al.*, *Lancet* **1982-I**, 640 (1982).
16. We thank H. Kanzaki for supplying umbilical cord blood specimens and K. Sugamura for helpful comments. Supported by grants-in-aid for cancer research from the Ministry of Education, Science, and Culture and the Ministry of Health and Welfare, Japan.

9 February 1982; revised 18 May 1982

Substance P: A Putative Sensory Transmitter in Mammalian Autonomic Ganglia

Abstract. *Repetitive presynaptic stimulation elicited slow membrane depolarization in neurons of inferior mesenteric ganglia from guinea pigs. This response was not blocked by cholinergic antagonists but was specifically and reversibly inhibited by a substance P analog, (D-Pro^2, D-Phe^7,D-Trp^9)-substance P, which also depressed the depolarization induced by exogenously applied substance P. The atropine-sensitive slow excitatory and slow inhibitory postsynaptic potentials evoked in neurons of rabbit superior cervical ganglia were not affected by the substance P analog. These and previous results provide strong support for the hypothesis that substance P or a closely related peptide is the transmitter mediating the slow depolarization. The latter may represent a sensory input from the gastrointestinal tract to neurons of the prevertebral ganglia.*

Synaptic transmission in autonomic ganglia involves several postsynaptic potentials with opposing polarities and time courses that range from a few milliseconds to several minutes (*1*). Acetylcholine, the classical transmitter in autonomic ganglia, acts on postsynaptic nicotinic and muscarinic receptors to give rise to fast and slow excitatory postsynaptic potentials (EPSP's), respectively. In a number of autonomic ganglia presynaptic stimulation evokes, in addition to cholinergic fast and slow excitatory potentials, a third excitatory potential that lasts for seconds or minutes. Since this slow depolarization is not blocked by cholinergic antagonists, it has been proposed that it is not mediated by acetylcholine (*2*).

Immunohistofluorescence, ultrastructural, biochemical, and electrophysiological studies (*3–8*) have implicated substance P (SP), an undecapeptide, as the transmitter mediating the slow, noncholinergic depolarization evoked in neurons of the guinea pig inferior mesenteric ganglia. Positive identification of SP as the transmitter in these ganglia has not been possible because of a lack of specific SP antagonists. Recently, several SP analogs have been developed that are antagonistic to SP in various tissues (*9*). We report here that SP analog (D-Pro^2,D-Phe^7,D-Trp^9)-SP (Pro, proline; Phe, phenylalanine; Trp, tryptophan), a differential antagonist of the effects of SP on smooth muscle (*9*), specifically and reversibly depresses noncholinergic transmissions in guinea pig inferior mesenteric ganglia.

Intracellular recordings were obtained from neurons of guinea pig inferior mesenteric ganglia and rabbit superior cervical ganglia (*5, 6, 10*). The ganglia were superfused with Krebs solution (*10*) to which appropriate concentrations of SP, SP analog, or other drugs were added.

Repetitive stimulation (30 Hz, 2 to 4 seconds) of hypogastric nerves elicited bursts of action potentials in the inferior mesenteric ganglia neurons. Most of these cells then underwent a slow depolarization (the noncholinergic depolarization). In some instances a prolonged hyperpolarization occurred subsequently (Fig. 1) (*6*). When applied in concentrations of 1 to 10 μM, the SP analog did not change the resting membrane potential or input resistance in 16 of 23 neurons tested (Fig. 1). In the remaining seven neurons, the analog caused a slow depolarization of several millivolts, accompanied in some instances by an increase in membrane resistance. The membrane potential generally returned to near the control level after continuous superfusion with SP analog for several minutes (Fig. 2).

The SP analog caused no measurable change in the amplitude of the initial fast EPSP's, whereas it reversibly depressed—in 13 of the 17 cells tested—the noncholinergic depolarization evoked by nerve stimulation. No appreciable change in the noncholinergic response was observed in the remaining four neurons. The depression of the depolarization induced by SP analog developed slowly; usually it reached a maximum 3 to 5 minutes after application of the substance was discontinued (Fig. 1). The amplitude of the depolarization gradually returned to the control level after the preparation was washed with Krebs solution for 15 to 30 minutes (Fig. 1).

The SP analog's depressant effect was related to its concentration. At concentrations of 1 and 10 μM, SP analog depressed the amplitude of the noncholinergic response 14 percent ($P < .05$, paired Student's t-test; $N = 6$) and 42 percent ($P < .01$; $N = 7$), respectively. At 50 μM, SP analog completely abolished the depolarization in less than 5 minutes in two cells tested; the effect was reversed after a 30-minute wash with Krebs solution. An initial augmentation of the depolarization was observed in 4 of the 13 neurons to which SP analog was applied; the enhancement was generally small (< 10 percent), and it was consistently followed by a much larger depression of the response. The prolonged hyperpolarization that follows the noncholinergic depolarization continued to be present when the application of SP analog attenuated or blocked the depolarization (Fig. 1).

Next we determined the effects of SP analog on membrane depolarization induced by exogenously applied SP. Membrane depolarization was elicited by ex-

0036-8075/82/0820-0739$01.00/0