LDH agent from CD/5 lymphoma thus differs from R.E.S. stimulating agent of sarcoma 180.

1. Riley, Vernon, Lilly, F., Huerto, E., Bardell, D., *Science,* 1960, v132, 545.
2. Riley, V., Wroblewski, F., *ibid.,* 1960, v132, 151.
3. Bailey, J. M., Stearman, M., Clough, J., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 148.
4. Notkins, A. L., Greenfield, R. E., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 988.
5. Yaffe, D., *Cancer Research,* 1962, v22, 573.
6. Old, L. J., Benacerraf, B., Clarke, D. A., Carswell, Elizabeth, Stockert, Elizabeth, *ibid.,* 1961, v21, 1291.
7. Wacker, W. E. C., Ulmer, D. D., Valee, B. L., *New Eng. J. Med.,* 1956, v255, 449.
8. Riley, Vernon, PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 751.
9. Notkins, A. L., Greenfield, R. E., Marshall, Diana, Bane, Louisa, *J. Exp. Med.,* 1963, v117, 185.
10. Plagemann, P. G. W., Watanabe, M., Swim, M. E., PROC. SOC. EXP. BIOL. AND MED., 1962, v111, 749.

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Temperature Induced Cellular Resistance to the "Lipovirus."* (28994)

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"Lipovirus" is a new transmissible cytopathic agent isolated from (and ostensibly present in) the acute phase blood of a patient with infectious hepatitis. It is capable of inducing marked DNA and thymine degradation in, and the release of a lipogenic toxin from, infected human cells(1,2,3). Electronmicrographic study reveals the presence of electron-dense ring structures of about 15 mu in diameter and their apparent aggregation to form spongiform bodies of about 100 mu as they migrate from the nucleus to the cytoplasm(4). Recent study shows that this agent is a new immunological entity, not related to most of the DNA-containing viruses or the myxoviruses capable of infecting man(5). Antibody to this agent has also been found to be prevalent in man, particularly in those patients suffering from a disease clinically diagnosed as infectious hepatitis(6). This paper describes the creation of a reverisble state of cellular resistance to the "lipovirus" by elevating the incubation temperature to 38.5°C.

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Materials and methods. Cell culture. Cell suspensions were prepared from cultures of permanent cell lines or from minced tissues by the standard trypsinization procedure. The cells were then grown directly on glass as a monolayer in nutrients consisting of 5% heated horse serum (56°C 30 minutes) in Eagle's basal medium(7), as modified in this laboratory(8). Unless otherwise specified, a permanent cell line derived from a human liver biopsy(9), referred to hereafter as the Lich-1 cell, was used in all experiments.

Temperature. Cultures to be maintained at 38-40°C or at 32-34°C were kept in standard laboratory incubators; those to be maintained at 36.5, 37.5, or 38.5°C were immersed in standard water baths, and those to be kept at 25°C were simply left at room temperature which fluctuated between 20 and 28°C. Temperatures in the incubators and water baths were measured with thermometers calibrated to 0.5°C. The thermometers were immersed in stoppered tubes filled with water, and readings were taken each morning when the incubators or water baths were opened for the first time.

"Lipovirus." A strain, which has undergone numerous passages through Lich-1 cells since its original isolation in 1954, was used. Inoculum for the present series of experi-

TABLE I. Onset and Progression of CPE in Infected Human Cell Cultures Kept at Different Temperatures.

Cell culture	Inoculum (ID_{50})	Temp 20–28	32–34	36.5	37.5	38.5	38–40
Lich-1	300	5–9*	3–5	5–10	6–9	none	none
	30		4–5	8–12	14–21		
	3		5–7	9–12	16–21		
Primary amnion	300	7–10	6–8	6–9	12–16	"	"

* 5–9 indicated that CPE was first recognized on the 5th day and was complete by the 9th day after inoculation. None indicated the absence of CPE after 21 days.

ments was prepared by subjecting infected Lich-1 cultures to one cycle of differential centrifugation as described(1). The product, which represented a 10-fold cencentration of infectivity originally present in the fluid phase of the culture, contained about $10^3 ID_{50}$ per ml.

Virus titration. Infectivity was assayed by the usual 10-fold serial dilution method, 4 cultures being used per dilution. The Lich-1 cell incubated at 32-34°C was used in all titrations. Inoculated cultures which failed to show specific cytopathic changes after 14 days were discarded as negative. The ID_{50} was calculated by the method of Reed and Muench. In 4 consecutive titrations by this method, values of 10^3, 10^3, $10^{3.5}$ and $10^{2.7}$ ID_{50} per ml were obtained for a virus pool.

Results. Onset and progression of specific cytopathic changes (CPE) in infected cultures kept at various temperatures. The results of 2 representative experiments, using cultures of human cells, are shown in Table I. The optimal temperature for the development of CPE was 32-34°C. At 36.5°C or 20-28°C, onset and progression of CPE was distinctly slower. Further suppression was noted at 37.5°C. At 38.5°C, CPE was completely suppressed. Similar results were obtained for primary chick embryonic cultures. It should be emphasized that there was no excessive cell degeneration in uninfected control cultures kept at 38.5°C.

Relation between virus synthesis and CPE. To determine if there is a positive correlation between the appearance of CPE and virus formation, the following experiment was performed. A series of replicate cultures were infected with about 300 infectious doses of "lipovirus"; after 2 hours at 33°C, the cultures were washed twice to remove unattached virus; 1 ml of nutrient medium was added to each culture; one or 2 cultures were assayed for infectivity and the remainder kept at 20-28, 32-34 and 38-40°C. The extent of CPE was recorded daily. Nutrient medium was not renewed; instead 1 ml of medium was added to each culture on the 5th day. Total infectivity or infective potential was determined on the 3rd and 5th or 8th day by converting the monolayer cultures into cell suspension prior to infectivity titration. In cultures showing complete CPE, cell suspension was prepared by vigorous shaking to detach the degenerated cells. In those with early or no CPE, 0.2% trypsin (Exp. 1) or 0.2% versene (Exp. 2) were used as the cell dispersing agent. This type of titration was adopted because infectivity remained chiefly cell associated(1). The results of 2 experiments are presented in Table II. The appearance of cytopathic changes was accompanied in all instances by significant increases in infectivity. At 38-40°C, neither cytopathic changes nor increase in virus content was noted.

Failure to demonstrate interferons from infected cultures kept at 38-40°C. The possibility that cellular resistance to the "lipovirus" at 38-40°C may be mediated through the release of interferons from infected cells was explored. The method of Ho and Enders (10) was used. Coxsackie B1, vaccinia, Sindbis and "lipovirus" at concentrations of 30-100 ID were used as challenge viruses. No interferon-like activity was demonstrated in supernatant fluid from "lipovirus"-infected culture incubated at 38-40°C. When a suspension of "lipovirus" was mixed with the supernatant fluid and kept at 38-40°C for

TABLE II. Correlation Between Extent of CPE and Infectivity Content of Infected Culture Kept at Several Temperatures.

Incubation temp	Day after infection	Infectivity and CPE per culture* Exp 1	Exp 2
32-34°C	0	2.0 (0)	2.3, 2.8† (0)
	3	2.5 (0)	5.8, 5.5 (+)
	5		6.0, 6.3 (++)
	7	6.3 (++)	6.0, 5.0 (++)
38-40°C	3	1.8 (0)	2.0, 1.8 (0)
	5		1.5, 1.5 (0)
	7	1.0 (0)	1.5, 1.0 (0)
20-28°C	3	1.5 (0)	
	7	3.8 (+)	

* Infectivity expressed as the negative logarithm of the highest dilution of the cell suspension which produced CPE in half of the inoculated cultures. (0), (+), and (++) indicated no, partial and complete CPE.

† Results of duplicate cultures.

24 hours, no loss in infectivity could be demonstrated.

Effect of temperature shift. Infected Lich-1 cultures which developed partial CPE at the optimal temperature regained their healthy appearance 3 to 7 days after transfer to the supra-optimal range (38-40°C). If the cultures were returned to the optimal range within 14 to 35 days, CPE would reappear. The interval between the transfer to the optimal temperature and the reappearance of CPE was, however, unusually long; it varied from 13 to 21 days in contrast to the interval of less than 6 days in cultures kept at the optimal temperature and infected with about 3 infectious doses. If the return to the optimal range was made after 55 days, CPE failed to reappear; but, such cultures were equally susceptible to reinfection. Thus, by shifting the incubation temperature, an infected Lich-1 culture may retain its infective potential for at least several months.

Influence of temperature on lethal action of "lipovirus" on chick embryos. The destructive action of the "lipovirus" on cultures of chick embryonic tissue in contrast to its innocuousness to whole embryos was emphasized in an earlier report(1). Since embryonated eggs were generally incubated at 37-39°C, a temperature unfavorable to the replication of this agent, experiments were performed to determine if the "lipovirus" was lethal to embryos kept at 33°C. The results are summarized in Table III. It was quite evident that 500 infectious doses of "lipovirus" given via the chorioallantoic, amniotic or allantoic routes were lethal to embryos kept at 33°C but not at 38°C. Embryonic death occurred regularly 4 to 7 days after infection. Evidence for virus replication was also quite conclusive. At least 10^4 infectious doses per ml were demonstrated in the amniotic or allantoic fluid collected from each of 20 eggs which died 4-7 days after infection. Embryos inoculated with medium control and incubated at 33°C showed a mortality of about 30%; the death of these embryos occurred, however, between the 4th to 14th day. When the "lipovirus" was given via the yolk sac, however, increase in embryo mortality was not consistently observed and replication of the agent could not be regularly demonstrated. When the "lipovirus" was incubated at 33°C for 24 hours with equal volume of yolk (from 7th and 14th day embryo), the original infectivity for Lich-1 culture was retained.

Discussion. The influence of temperature on virus replication has been quite extensively studied(11-13). The results obtained for the "lipovirus" differed strikingly from those described by other investigators. Com-

TABLE III. Effect of Incubation Temperature on Mortality of Chick Embryos Injected with the "Lipovirus".

Route of inoculation	Inoculum	Embryo mortality* at 33°C	38°C
Amniotic	500 ID	24/24	2/16
"	medium control	9/20	
Allantoic	500 ID	7/8	0/8
"	medium control	0/8	
Chorioallantoic Membrane	500 ID	18/18	0/16
	medium control	3/14	
Yolk sac	500 ID	16/44	1/10
" "	medium control	12/42	

* Seven-day embryos were used for amniotic and yolk sac inoculations, 10-day embryos for allantoic and chorioallantoic inoculation. Embryos found dead within 72 hours after inoculation were excluded from tabulation. Mortality was expressed as number dead/inoculated. All experiments were arbitrarily terminated after embryos reached 21st day of incubation.

plete suppression of viral replication occurred in infected cultures at 38.5°C and such cultures retained their infectious potential for at least 35 days. This is of interest because this critical temperature is only slightly higher than the physiological temperature of man and is not detrimental to the survival or multiplication of human cells *in vitro*. Thus, a relatively minor alteration in cell environment may bring about a complete reversal of cell susceptibility to this very unusual transmissible agent. The mechanism for this complete reversal of cellular susceptibility is not known. It is not due to the release of interferon-like substances or extracellular inhibitors, or due to an increase in the rate of extracellular viral decay. There is no evidence that cellular metabolism was drastically altered. The state of resistance is reversible and, therefore, not due to the emergence of a resistant cell population.

This temperature effect appears to be equally applicable to the lethal action of this agent for chick embryo. Embryos infected via the chorioallantoic amniotic or allantoic route died regularly 4-7 days after infection when incubated at 33°C but not at 38°C. The failure of the "lipovirus" to kill embryos when given via the yolk sac remains unexplained. It is interesting that an agent with a hypothetical lipid-rich outercoat(1) fails to multiply regularly when deposited in a lipid-rich cavity of the chick embryo.

The finding that 32-34°C rather than 37°C is optimal for the replication of "lipovirus" has vastly expedited the production of "lipovirus"-infected cells. Such cells are needed in large quantity for preparation of "lipovirus" antigen(6) and for investigation of enzymatic changes which lead to the degradation of host DNA(3) and accumulation of lipid(2). The finding that specific CPE would develop in infected cultures kept at 20-28°C also simplifies the titration of "lipovirus" infectivity. Cultures kept at room temperature remain relatively healthy for 14 days without nutrient renewal. The tedious step of feeding the large number of cultures used in a titration may thus be eliminated.

Summary. The optimal temperature for development of specific degeneration in "lipovirus"-infected cultures was 32-34°C. Partial and complete suppression was noted at 37.5°C and 38.5°C respectively. The "lipovirus" was lethal to chick embryos if the virus was inoculated into the amniotic or allantoic sacs or onto the chorioallantoic membrane and the embryos then incubated at 33°C. Inoculation into the yolk sac or elevation of incubation temperature to 38°C nullified the lethal action of the "lipovirus" on chick embryos.

1. Chang, R. S., Humes, M., *J. Exp. Med.*, 1962, v115, 937.
2. Chang, R. S., Geyer, R. P., Andrus, S. B., *ibid.*, 1962, v115, 959.
3. Chang, R. S., Liepins, H., *ibid.*, 1962, v115, 967.
4. Dunnebacke, T. H., *Virology*, 1963, v21, 203.
5. Chang, R. S., *J. Immunol.*, 1964, v92.
6. Chang, R. S., Owens, S., *ibid.*, 1964, v92.
7. Eagle, H., *Science*, 1955, v122, 501.
8. Chang, R. S., *J. Exp. Med.*, 1961, v113, 405.
9. ———, PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.
10. Ho, M., Enders, J. F., *Virology*, 1959, v9, 446.
11. Walker, D. L., Boring, W. D., *J. Immunol.*, 1958, v80, 39.
12. Lwoff, A., *Bact. Rev.*, 1959, v23, 109.
13. Isaacs, A., *Cold Spring Harbor Symposium on Quantitative Biology*, 1962, v27, 343.

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