A PROGRESS REPORT

to

U. S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

STUDIES ON VIRUS-INDUCED CELL FUSION

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Progress Report

We are studying the mechanism of cell fusion induced by fusion-causing mutants of Herpes Simplex Virus (HSV) Type I. We have isolated a number of these mutants which, unlike the wild type strain KOS, cause extensive cell fusion during an otherwise normal infection. The mutants are designated syn because of their ability to cause the formation of syncytia, cells each containing many nuclei, during infection. The mutation is not conditional-lethal, but rather should be thought of as a plaque morphology mutation. A year ago we proposed to develop a simple and quantitative assay for cell fusion, to determine the glycolipid content of wild type infected cells, syn mutant infected cells, and uninfected cells, and to study membrane fluidity by determining rotational correlation times of spin label molecules in wild type infected cells, syn mutant infected cells and uninfected cells.

We noted last year that in developing an understanding of the molecular mechanism of cell fusion it would be important to employ an assay for cell fusion that would rapidly and reproducibly yield a number that could be used as an index for the amount of cell fusion that had occurred for a particular set of conditions. We hoped we could extract from the number, the probability that a single cell would fuse per unit time. We have developed a suitable assay for cell fusion employing a Coulter counter to measure the disappearance of single cells as a function of time after infection. Human embryonic lung cells growing on a glass surface were infected with about four plaque-forming units per cell. At various times after infection the monolayer of cells was harvested with a trypsin-EDTA solution which separates cells. We adjusted the Coulter counter to record only the number of small single cells. By this assay cells were considered to be unfused if they were separated into small single cells by the harvesting procedure. For this assay the number

of single cells remained constant until 5.3 hours after infection (the onset of fusion) and then decreased exponentially with time. The number of single cells decreased to 10% of the original number 10.2 hours after infection. The slope of this line is the product of the probability ^{per unit time} that a single cell will fuse and the initial concentration of single cells. Data are given in a preprint ~~included as Appendix 1.~~ In that appendix we show that data obtained with this assay are the same as those obtained using a microscope, and that the rate of fusion is dependent on the multiplicity of infection and cell concentration. We have also shown that syn mutant infected cells can fuse with either uninfected cells or other syn mutant infected cells. However, we have recently noted that fusion does not occur between syn mutant infected cells and wild type infected cells, perhaps indicating the presence of an inhibitor in the wild type infected cells (data not shown).

We include as Appendix 2 a derivation of equations for the disappearance of single cells as a function of time after infection. These equations predict, for the assumptions used, that single cells will disappear exponentially with time. They also indicate that, to a first approximation, one fusion event results in the disappearance of one cell.

Although the Coulter counter assay is completely satisfactory, we are also developing a second assay for cell fusion based on dipole-dipole energy transfer from one fluorescent dye molecule to another. Two populations of cells are used, one labeled in its membranes with fluorescein and the other with rhodamine. The dyes have been covalently linked to 18 C hydrocarbon chains so that they will partition into plasma membranes. We abbreviate the dyes F18 and R18 for the fluorescein and rhodamine fluorescent hydrocarbon probes, respectively. Two conditions are required for dipole-dipole energy transfer between fluorescent dyes. First, the dyes must be close to

each other (<50A) and, second, the emission spectrum of one dye must overlap the absorption spectrum of the other dye. Both the distance dependence and the spectral overlap for dye molecules in solution have been extensively studied by Forster (1960). The emission spectrum of F18 overlaps the absorption spectrum of R18. If the two molecules are sufficiently close, as they might be when an F18-labeled cell fuses with an R18-labeled cell, there could be an enhancement of R18 fluorescence due to energy transfer from F18 molecules.

Briefly the experimental procedure is as follows. Cells are labeled with F18 and other cells with R18 by adding the fluorescent probes to the growth medium. After 12 hours viruses are adsorbed to the labeled cells. Following virus adsorption the cells are harvested, F18 and R18-labeled cells mixed in equal proportions, and seeded in petri dishes in sufficient quantity to produce a confluent monolayer upon cell attachment. Fluorescence is periodically recorded using a fluorometer with an exciting wavelength of 460 nm and determining fluorescence at 600 nm. We observe an increase in fluorescence that is coincident with the onset of fusion using syn mutant infected cells. The increase is not observed with uninfected cells, wild type infected cells, or syn mutant infected cells in which fusion has been blocked by 2-deoxy-D-glucose. Until now we have used a Coulter counter to measure cell fusion. The fluorescent enhancement technique has the advantage that cells do not have to be harvested in order to make a measurement on the extent of fusion. Therefore, an entire fusion kinetics curve can be obtained from repeated measurements on cells in a single petri dish. We are also hopeful that we will be able to adapt the fluorescent enhancement assay to measure the fusion of vesicles prepared from cell lysates. This cannot be done with the Coulter counter assay.

Experiments to determine if differences exist in the glycolipid composition of wild type infected cells, syn mutant-infected cells and uninfected cells are in progress. These studies have gone much slower than we anticipated, mainly because the glycolipids comprise only about 2% of total lipids. As a result these studies will be extended into the coming year (see Grant Proposal). However, as a prelude to the glycolipid determinations we did examine the phospholipid profiles of wild type infected, syn mutant infected and uninfected cells. Some data are shown in Appendix 3. We note that although there are some differences in the phospholipids extracted from infected cells relative to uninfected cells, there are no obvious differences in the phospholipids extracted from syn mutant infected cells relative to wild type infected cells.

Experiments employing electron spin resonance and spin labels are very incomplete. Nonetheless, they should be finished by the time the current grant expires. However, in conjunction with Drs. Snipes and Keith we have studied the lethal effect of butylated hydroxy toluene (BHT) on non-enveloped and enveloped bacterial and animal viruses. BHT is an anti-oxidant commonly used as a food preservative. It is water insoluble and partitions into membranes. We found that BHT specifically inactivates enveloped viruses, perhaps by altering the membrane structure so as to prevent virus adsorption and/or entry into the host cell. This study has just appeared in SCIENCE and a preprint is enclosed as Appendix 4.

The publication of our former work on tritium decay is still not completed but should be by the time the current grant expires. A preprint of the comparative mutagenic effect of adenosine-2-³H relative to guanosine(adenosine)-8-³H, and of thymidine-6-³H relative to thymidine-methyl-³H decays is ~~included as Appendix 5.~~ Two other manuscripts exist in draft form and we anticipate

writing a review of the tritium research during the calendar year. ~~We also~~
include, as ~~Appendix~~, an article on UV mutagenesis and repair in E. coli
which has been accepted for publication in Genetics.

Forster in Comparative Effects of Radiation, ed. by Burton, Kirby-Smith and
Magee, Wiley, N.Y., 1960. p.300

Publications

W. Snipes, S. Person, A. Keith & J. Cupp. Butylated hydroxytoluene inactivates lipid-containing viruses. Science 188:64-66 (1975). COO-3419-15

S. Person, J.A. McCloskey, W. Snipes & R.C. Bockrath. Ultraviolet mutagenesis and repair in an Escherichia coli strain containing a nonsense codon. Accepted for publication in Genetics. COO-3419-5

S. Person, W. Snipes & F. Krasin. Mutation production from tritium decay: a local effect for ^3H 2-adenosine and ^3H -thymidine decays. Submitted to J. Mol. Biol. COO-3419-16

S. Person, R.W. Knowles, G.S. Read, S.C. Warner & C.V. Bond. The kinetics of cell fusion induced by a syncytia-producing mutant of Herpes Simplex Virus Type I. Submitted to Virology. COO-3419-12

F. Krasin, S. Person & W. Snipes. The chemical product of cytosine-5- ^3H decay. MS in preparation.

F. Krasin, F. Hutchinson, R. Ley & S. Person. Cross-linking and single strand breaks, and lethality from tritium decay: Local effects for adenosine-2- ^3H decays. MS in preparation.

Related Research

R. Knowles & S. Person. The effects of 2-deoxyglucose, glucosamine and mannose on cell fusion and the glycosylation of glycoprotein with a syncytial mutant of Herpes Simplex Virus. MS in preparation.

P.R. Morse II, M. Ruhlrig, W. Snipes & A.D. Keith. A spin-label study of the viscosity profile of sarcoplasmic reticulum vesicles. Accepted for publication in Arch. Biochem. Biophys. (M.R. is graduate assistant previously supported by this grant.)

- A. The disappearance of single cells as a function of time after the onset of fusion

Let N_1 be the number of single or mononucleated cells at any time, t

N_2 " " " " binucleated cells at any time, t

N_3 " " " " trinucleated " " " " "

etc.

N_2 is formed from N_1 according to

and N_3 is formed from N_1 and N_2 according to

and N_4 is formed from N_1 and N_3 according to

etc.

The rate constant for the formation of a binucleated cell is designated k_1 , for the formation of a trinucleated cell k_2 , etc.

The rate of disappearance of single cells then is given by

$$\frac{dN_1}{dt} = -k_1[N_1][N_1] - k_2[N_1][N_2] - k_3[N_1][N_3] - \dots \quad (4).$$

If we assume that a binucleated cell fuses with twice the probability as a mononucleated cell, that is $k_2 = 2k$, and similarly $k_3 = 3k$ and $k_1 = k$, the equation becomes

$$\frac{dN_1}{dt} = -k[N_1][N_1] - 2k[N_1][N_2] - 3k[N_1][N_3] - \dots \quad (5)$$

$$\text{or } -k[N_1] \left\{ [N_1] + 2[N_2] + 3[N_3] + \dots \right\} \quad (6)$$

but the quantity in brackets {} is N_1^0 , the total number of nuclei at any time t , which is also equivalent to the number of single cells at time $t = 0$.

Substituting N_1^0 gives

$$\frac{dN_1}{dt} = -k N_1^0 N_1 ; \quad (7)$$

rearranging gives

$$\frac{dN_1}{N_1} = -k N_1^0 dt ; \quad (8)$$

integrating gives

$$\ln N_1 = -k N_1^0 t + C ; \quad (9)$$

substituting the boundary conditions at $t = 0$, $N_1 = N_1^0$ gives

$$\ln \frac{N_1}{N_1^0} = -k N_1^0 t , \quad (10)$$

or in exponential notation

$$\frac{N_1}{N_1^0} = e^{-k N_1^0 t} . \quad (11)$$

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We conclude, for the assumptions used, that the disappearance of single cells is exponentially related to time. We recall that the assumption was that a binucleated cell fuses with a single cell with a probability that is twice the probability with which two single cells will fuse. Similarly, we assumed that a trinucleated cell fused with a single cell with a three-fold higher probability, etc. If plasma membranes and fusion factors are conserved during fusion, the assumption is valid to a first approximation. We also note that the above derivation does not include a consideration of fusion between multinucleated cells.

- B. The rate of disappearance of small single cells as a function of time after onset of fusion

We actually do not measure the disappearance of all single cells in the Coulter counter assay but only the disappearance of the small single cells in the threshold interval 10-15 (32% of the population).

In this case all the symbols will be as before except we will split single cells into those inside the interval (N_1^{in}) from those outside the interval (N_1^{out}). Then the rate of disappearance of single cells in the interval is given by

$$\begin{aligned} \frac{dN_1^{in}}{dt} = & -k_1 [N_1^{in}] [N_1^{in}] - k_1 [N_1^{in}] [N_1^{out}] \\ & -k_2 [N_1^{in}] [N_2] - k_3 [N_1^{in}] [N_3] - \dots \end{aligned} \quad (12)$$

and assuming $k_3 = 3k_1$, $k_2 = 2k_1$, $k_1 = k$, ... as before,

$$\frac{dN_1^{in}}{dt} = -k [N_1^{in}] \left\{ [N_1^{in}] + [N_1^{out}] + 2[N_2] + 3[N_3] + \dots \right\} \quad (13)$$

$$= -k [N_1^{in}] N_1^o \quad (14);$$

since $N_1^{in} + N_1^{out} = N_1$

integrating and substituting gives

$$\frac{N_1^{in}}{N_1^{in(o)}} = e^{-k N_1^o t} \text{ as before.} \quad (15)$$

- C. The rate of occurrence of fusion events as a function of time after the onset of fusion

Every time a cell inside the threshold interval fuses with a cell outside the interval (one fusion event) a single cell disappears from the threshold.

In this case there is a one-to-one relationship between the number of

fusion events and the number of single cells that disappear from the threshold interval. When fusion occurs for two cells, both inside the threshold interval, a single fusion event causes the disappearance of two single cells from the threshold interval. Therefore, the differential equations for the rate of disappearance of single cells are also valid for the rate of occurrence of fusion events, except that the first term^{of} (12) must be divided by two to allow for the disappearance of two cells due to a single fusion event. Letting F^{in} be the number of fusion events at any time, t , in the threshold interval 10-15

$$\begin{aligned} \frac{dF^{in}}{dt} = & -\frac{k_1}{2} [N_1^{in}] [N_1^{in}] - k_1 [N_1^{in}] [N_1^{out}] \\ & - 2k_1 [N_1^{in}] [N_2] - 3k_1 [N_1^{in}] [N_3] - \dots \end{aligned} \quad (16)$$

giving

$$\frac{dF^{in}}{dt} = -k [N_1^{in}] \left\{ \frac{[N_1^{in}]}{2} + [N_1^{out}] + 2[N_2] + 3[N_3] + \dots \right\}. \quad (17)$$

If we add and subtract $[N_1^{in}]/2$ to the right side of the equation we get

$$\frac{dF^{in}}{dt} = -k [N_1^{in}] \left\{ [N_1^{in}] + [N_1^{out}] + 2N_2 + 3N_2 + \dots - \frac{[N_1^{in}]}{2} \right\}; \quad (18)$$

substituting $N_1^o - \frac{N_1^{in}}{2}$ for the quantity in brackets we get

$$\frac{dF^{in}}{dt} = -k [N_1^{in}] \left(N_1^o - \frac{N_1^{in}}{2} \right). \quad (19)$$

Rewriting (14)

$$\frac{dN^{in}}{dt} = -k N_1^{in} N^o; \quad (20)$$

therefore the rate of disappearance of single cells per fusion event is

$$\frac{dN_1^{in}}{dF_1^{in}} = \frac{kN_1^{in} N_1^o}{kN_1^{in} \left(N_1^o - \frac{N_1^o}{2} \right)} \quad (21)$$

$$= \frac{N_1^o}{N_1^o - \frac{N_1^{in}}{2}} \quad (22)$$

Rearranging (dividing top and bottom of right hand side by N_1^o)

$$\frac{dN_1^{in}}{dF_1^{in}} = \frac{1}{1 - \frac{1}{2} \left(\frac{N_1^{in}}{N_1^o} \right)} \quad (23)$$

N_1^{in}/N_1^o = fraction of total cells that exist as single cells in the threshold interval 10-15 at any time, t .

At $t = 0$ this fraction = 0.32 (see Fig. 1, Appendix 1) giving

$$\frac{dN_1^{in}}{dF_1^{in}} = \frac{1}{1 - 0.16} = \frac{1}{0.84} = 1.19. \quad (24)$$

At long times N_1^{in}/N_1^o approaches 0 so that

$$\frac{dN_1^{in}}{dF_1^{in}} \sim 1. \quad (25)$$

We conclude that, at the onset of fusion and for the conditions of the experiments, a single fusion event results in the disappearance of 1.19 cells from the threshold interval 10-15 (Equation 24), and that later in fusion this number approaches 1.0 (Equation 25).

Figure Caption

Developer A - Vertical Direction - 65:35:5 Chloroform-Methanol-7 N. Ammonium Hydroxide (developed twice).

Developer B - Horizontal Direction - 10:4:2:2:1 Chloroform-Acetone-Methanol-Acetic Acid-Water

Human Embryonic Lung Cells were seeded at a density of 5×10^4 cells/cm² in 2 ounce culture bottles and incubated at 37° C. for 40 hours until almost confluent. Cell layers were washed with TBS buffer and infected with either KOS wild type Herpes virus or the SYN 20 cell-fusing mutant. Control cells were treated with an equivalent volume (0.2 ml) TBS. After an adsorption time of 1 hour, virus suspensions were removed and fresh medium was added. After incubation at 34° C. for 5 hours 250 microcuries of carrier-free $H_3^{32}PO_4$ was added to each sample. At 10 hours after adsorption cell layers were washed with TBS and scraped off successively with 8 ml chloroform and 4 ml methanol. 3 ml water (Folch Extraction) was added with vortexing, and the layered suspensions were allowed to stand overnight at -26° C. under nitrogen atmosphere. The lower, organic, phase was removed with a pipette, and the upper phase was washed once with 8 ml of theoretical lower phase¹ (86:14:1 chloroform-methanol-water). The combined lower phases were dried under nitrogen and the residue was applied to a 20 x 20 cm. silica gel thin layer plate (Analtech, Inc.) in approximately 50 microliters of 2:1 chloroform-methanol. The plates were developed in two dimensions as above and autoradiographed for 48 hours. In the upper left corner can be seen lipid spots which appear in the uninfected cells, but not in the two virus-infected cell extracts.

¹ J. Folch, M. Lees, G. H. Sloane-Stanley, "A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues." J. Biol. Chem. 226:497 (1957).

Wild-Type Infected Cells

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Syn-20 Infected Cells

Uninfected Cells