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TECHNICAL MANUSCRIPT 401

GENETIC CONTROL OF VIRUS PRODUCTION IN CELL LINES

Jerry S. Walker
Ralph E. Lincoln
Francis J. Weirether

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TECHNICAL MANUSCRIPT 401

GENETIC CONTROL OF VIRUS PRODUCTION IN CELL LINES

Jerry S. Walker

Ralph E. Lincoln

Francis J. Weirether

Process Development Division
AGENT DEVELOPMENT AND ENGINEERING LABORATORY

Project 1C522301A059 January 1968

ABSTRACT

A procedure to maintain genetic control during virus production in tissue cell lines is proposed and discussed. Both the tissue cell in which the virus product replicates and the virus inoculum must be homogeneous in order to produce a virus with the expected characteristics. Certain philosophical and technical aspects of the problem are discussed in relation to developing and maintaining genetically homogeneous stock culture, inoculum, and product.

I. INTRODUCTION

Evidence from molecular genetics shows that variation of cell lines and viruses is similar to that of bacteria, fruit flies, and maize. For example, it is attributable either to changes in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) for RNA-type viruses or to changes in the environment that allow previously unobserved phenotypic expression.[*,1] Variation in genotype or environment of either the cells or the virus may result in an altered interaction that affects the virus progeny qualitatively or quantitatively. The end product is the result of two biological systems, and it is necessary, therefore, to maintain genetic and physiochemical control over both systems, the cell and the virus, in order to maintain uniformity of the process and thus of the product.

This paper describes methods for controlling the production of a virus that uses cell lines as a host and tests to ascertain or assure the quality of the product. From the system described, one may derive principles useful for the control and production of viruses in other systems.

II. PRESENT SITUATION

A. MAMMALIAN CELL STOCKS

Although there are exceptions, the present quality standards for stock cultures of mammalian cells are generally low. For example, many cell lines discussed in published studies contain latent or inapparent viruses,[2] pleuropneumonia-like organisms (PPLO),[3] and bacteria.[4] Antibiotics in the growth medium control contaminants to some degree; in most cases contaminants become evident when growth is attempted in antibiotic-free medium. The presence of latent viruses may not be apparent unless the environment is changed or the cellular material is examined by electron microscopy.

The species source of some cell lines is no longer correctly identified. At present, most cytogenetic studies are concerned with chromosome number and morphology. However, very few, if any, mammalian cell lines can be characterized by a single test. Furthermore, few lines have been cloned and characterized for genetic homogeneity. Frequently, cell lines are subcultured continually on an irregular schedule using nonstandardized growth media and thereby allowing genetic variation to originate and be retained in the culture.

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* Walker, J.S.; Parker, F.; Merchant, D.J. Unpublished data.

B. VIRAL STOCKS

Quite probably there is no genetically homogeneous viral stock in use today. In deriving viral stocks, replication of the virus depends upon the physiological system of the host. The biological variations in such systems may, therefore, result in heterogeneity in the viral progeny. In the chick fibroblast system, for example, cells of unknown origin from all three germinal layers are present. These cells possess different enzymatic and physiological capabilities and are in various stages of differentiation. As a result, the virus produced may reflect the heterogeneity of the cells in which it replicates.

With a few possible exceptions, we do not know how many particles constitute an infectious unit. We do know that with some viruses, both complete and incomplete viral particles occur. Because in phage-bacterial systems defective viral units may complement each other and viral recombination can occur, there is a biological basis for assuming that these phenomena may occur with mammalian viruses. There are, in fact, some reports of these phenomena.[5,6]

Plaque size is commonly accepted as a genetic marker for certain viruses. Our experience with Venezuelan equine encephalomyelitis* showed that we were unable to stabilize plaque size. This observation appears to be common with other workers[7] and not unexpected, because plaque size is dependent on several interacting factors.[8] Although other parameters such as antigenic differences, thermostability, and urea sensitivity have been used as genetic markers, we do not feel that these characters are discriminatively useful for selection because the character cannot be maintained. A case in point is the T markers of poliovirus. In any case, the standards for homogeneity should approach those used by other microbiologists.

III. PROCEDURES

To minimize the foregoing deficiencies and difficulties the following basic procedures are proposed.

A. BASIC OUTLINE

As indicated in Figure 1, from the selected strain of both the cell line and the viral stock, a single viable unit (i.e., a single cell of the cell line or a plaque-forming unit of the virus) is obtained and grown to

* Unpublished data.

the quantity necessary for preparation of a stock culture. The possibility of selecting genetic mutants during the buildup is minimized by maintaining logarithmic growth of the cells under optimal conditions for the desired clone. This lessens selective pressures that might allow variants to proliferate and thus be selected.

For stabilization and preservation a procedure is employed that results in high initial recovery and maintenance of viability through the period of anticipated use, e.g. 5 to 25 years. The suitability of the clonal stock is proven and a quantity of it is set aside as the proven primary stock culture; it may be used at designated intervals to produce a secondary stock that, in turn, will produce the required inoculum. The possibility of uncontrolled genetic changes is minimized by returning to the proven primary stock. Each secondary seed stock also is tested for its ability to produce the desired product, is used for a designated time, and is then replaced with another secondary inoculum derived directly from the primary seed stock. These successive steps are designed to maintain the homogeneity of the primary stock during production. It should be noted that acceptance of an inoculum is based on two discriminating tests: one before acceptance of the stock culture as a proven one, the second on acceptance of the secondary stock. A third optional test also may be made on the inoculum itself.

B. PROCEDURE OF CHOICE

1. Desirable Standards

Personal experience indicates that the quality of an operation deteriorates with use and time, so a laboratory must deliberately set high standards of operation and quality assurance. For example, the risk of contamination and overgrowth of one cell line by another can be virtually eliminated by using only one cell line in a laboratory. Even with this restriction, our laboratory conducts monthly fluorescent antibody tests to document the species purity of cell lines. In addition, monthly tests prove freedom from contamination with PPLO, bacteria, and molds. If tests are positive for contaminants, all material is destroyed and new inoculum is prepared from a pure primary or secondary stock culture, as the case may be. No antibiotics are used because they increase the probability of masking PPLO and other contaminants. Use of laminar flow cabinets and/or white rooms is, in our experience, desirable for tissue culture production when antibiotics are not used in the growth medium. Many other workers have also proved that antibiotic-free cell lines can be continued indefinitely without contamination.

Primary Stock Culture

Selected Strain → Single Viable Unit → Build-up → Concentration → Stabilization and Preservation → Testing and Proving → Acceptance → Storage → Mass Transfer (Secondary Stock Culture)

Secondary Stock Culture

Mass Transfer → Buildup → Stabilization or Maintenance → Proving Tests → Acceptance → Storage → Mass Transfer (Inoculum)

Inoculum

Mass Transfer → Inoculum Buildup → Tests Optional → Use as Inoculum Product → Tests

Figure 1. General Scheme for Seed Stock Maintenance and Use.

2. Initial Stock Selection

In selecting a cell line, one must evaluate its efficacy in terms of viral spectrum, generation time, quantity of cells and/or virus produced per ml of medium used, and medium complexity and cost. Some of these factors also can be used to screen clones.

3. Environmental Control

Growth environment is currently controlled by selection of the medium and continuous control of pH, oxidation-reduction potential, CO_2, and O_2. Instrumented New Brunswick fermentors or glass chambers of about 1 liter capacity are used to grow the inoculum and final product.[9] Although there is still much to be learned about optimal physicochemical environments, it is apparent that genetic expression will be less subject to undesirable selective pressures in a controlled environment.

4. Cloning and Establishing a Stock

All stocks and inocula are initiated from single-cell clones. A plating efficiency of at least 50% is desirable to minimize the possibility of genetic selection. For the L-M cell, it is necessary to use a medium containing 20% (v/v) sterile, filtered horse serum to obtain a plating efficiency above approximately 30%.* The locations of single cells are marked after observation with a microscope, and the clones are picked 7 to 10 days after plating while the colony is still increasing in size. The selected colony is placed in 1 ml of medium in a test tube and held in a CO_2 incubator for 5 days. The resulting cell growth is transferred to a Falcon T-30 flask containing 5 ml of medium from which either suspension cultures or monolayer cultures in Falcon T-60 flasks may be prepared. Stocks are frozen at the time of genetic characterization to eliminate the possibility of changes occurring upon further culture.

5. Preservation of the Stock Culture

Cells are grown in suspension to a concentration of 1.5×10^6 to 2.0×10^6 cells/ml and centrifuged at 4 C. The packed cells are resuspended to one-tenth of the original volume in medium containing 5% glycerol or dimethyl sulfoxide, pH 7.0. One-ml portions of cell suspension are placed in 2-ml vials and frozen in liquid nitrogen at the rate of 1 C/min through the range 4 to -50 C and then plunged into a liquid nitrogen storage tank. To obtain high recovery, it is necessary to work rapidly and to maintain refrigerated conditions (4 C) from the time centrifugation is started until the cells are frozen.

* R. Carter et al., unpublished data.

6. Reinitiation of Growth

One vial of frozen stock is thawed and the cells are transferred to 100 ml of medium in a 250-ml flask that is placed on a reciprocating or rotary shaker. Gaseous balance is maintained by rubber stoppers for 24 hours, after which some diffusion through a needle is allowed. The method and equipment have been described by which cultures can be grown uniformly and consistently to concentrations of 1.5×10^6 to 2.0×10^6 cells/ml in 100-ml cultures in 7 days from the frozen stock.[10]

7. Proving Primary Stock Cultures

At least 200 vials of stabilized and preserved stock are produced from each clone. Usually five tubes are used in tests to determine if the quality of the stock is acceptable. The acceptance tests are outlined in Figure 2.

Reconstitute five tubes/clone
Transfer to 100 ml medium in 250-ml flasks
Agitate. Grow to 1.5×10^6 to 2.0×10^6 cells/ml
Inoculate new growth flask with 500,000 cells/ml
Serially transfer three more times

<u>Tests</u>:

- Doubling time for each 24 hours and overall
- Viable cell count
- Contamination: other cell lines, bacteria, PPLO, and virus
- Genetic variation
  - Clonal type
  - Alkaline phosphatase
  - Zymogram analysis
- Virus production
- Other discriminating tests

<u>Standards</u>:

Standards need to be established that are specific for each clone in the growth environment being used. Standards should guarantee maintenance of genetic homogeneity and high quality of product.

Destroy stocks not meeting all specifications.
Identify and accept clonal stock as primary stock culture.

Figure 2. Acceptance Tests and Standards for the Primary Stock Culture.

8. Preparation of Secondary Stock Cultures

A single vial of proven primary stock culture is used to prepare a secondary stock from which the individual inocula used during a 6-month period are prepared. The culture is reconstituted, grown to the volume required, and preserved by the same procedures used with the primary stock culture. Although the same tests are used for the primary and the secondary stock cultures, specifications for the primary and the secondary stock may be different. One vial of proven secondary stock is used to prepare each process run. A lead time is required to prepare secondary seed and to conduct the quality assurance tests. The time needed will depend upon the tests conducted, because immunizing and/or virulence tests on a virus product may require an extended period. The use of a mass transfer, rather than cloning, varies from our process with bacteria in which clones are used at both the secondary and inoculum steps[11] but is employed because of the long generation time of mammalian cells.

9. Viral Stocks

Currently, it is technically impossible to select and maintain a genetically homogeneous virus stock. That any procedure will produce a clone originating from a single viral particle cannot reasonably be proved; moreover, the morphological and biochemical markers available today have little, if any, genetic meaning or discriminative value. Nevertheless, it is necessary to attempt to minimize viral variation by following certain procedures:

1) Select and use single-plaque isolates.

2) Propagate virus to the required titer and volume in a cell system such as that just described by a series of transfers, rather than by obtaining virus from a culture produced over a long period or by repeatedly harvesting virus from the same culture. This procedure, we believe, decreases the chance of selecting for a less virulent variant from a virulent stock,[12] because avirulent variants may not lyse the cell but rather may be released continuously.[13]

3) Clarify, stabilize, and preserve by procedures that maintain high viability. If the virus is to be frozen, only 10% (v/v) skim milk need be added after clarification. However, if the virus is to be preserved as a lyophilized stock, the stabilizer must be more specifically tailored to the specific requirements of each virus.

4) Compare virulence, immunogenicity, or other cogent characteristics by various routes of inoculation in several hosts.

To reiterate, we are working without the required knowledge of viral genetics. The procedures employed were developed on the premise that viral inheritance is essentially like that of other organisms and that the use of these procedures will "minimize" variation.

IV. FUTURE WORK

Better control of the genetics of mammalian cells will be gained when isoenzyme and other enzyme systems are more fully understood. From preliminary work in our laboratory with selected isoenzymes it appears possible to distinguish between the L-M and L-DR lines as well as among a number of clones of these lines (Table 1).* No single enzyme is a suitable marker for all the clones; however, by using a combination of isoenzymes, we find different patterns emerge for each clone. Further development of this method will allow not only identification of each stock, but also detection of genetic change in a stock. Because at present the significance of the Type A, B, and C particles found in some L cultures has not been evaluated, a record of these particles should be kept on all cell stocks. For viral stocks, techniques must be developed that will allow isolation of viral mutants independent of living systems. Selection based on (i) altered base composition of the viral DNA and/or RNA, (ii) use of altered electrical charges of the viral particles that reflect changes in amino acid content, and (iii) alteration of the active attachment site of the capsomer are promising methods of approach. However, at the present pace of work in molecular biology, other approaches will soon become attractive.

* Unpublished work from this laboratory.

TABLE 1. HYPOTHETICAL DISTRIBUTION OF ENZYMES IN CELL LINES

Enzyme Quantitated	Cell Line: L-M	L-DR	Clone 1-2 of L-Ma	Clone 1-7 of L-Ma
Isoenzyme 1	A		A	A
Bands A, B, & C	B	B	B	
	C	C	C	C
Isoenzyme 2	A	A	A	No bands
Bands A & B		B		
Isoenzyme 3	A	A	A	A
Bands A, B, C, & D	B	B		B
	C	C	C	
	D	D	D	D

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13. ABSTRACT

A procedure to maintain genetic control during virus production in tissue cell lines is proposed and discussed. Both the tissue cell in which the virus product and the virus inoculum must be homogeneous in order to produce a virus with the replicates and the virus inoculum must be homogeneous in order to produce a virus with the expected characteristics. Certain philosophical and technical aspects of the problem are discussed in relation to developing and maintaining genetically homogeneous stock culture, inoculum, and product.

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*Genetics
*Cells
Replication
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