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Agriculture Handbook No. 404

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PROCUREMENT SECTION
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PROCEDURES FOR THE IN VITRO ASSAY
OF VIRUSES AND ANTIBODY
OF AVIAN LYMPHOID LEUKOSIS
AND MAREK'S DISEASE

Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE

Foreword

The methods outlined herein were originally compiled from instructions prepared in 1962 at the Regional Poultry Research Laboratory (RPRL), East Lansing, Mich. In 1964 these instructions were revised and expanded. However, development of newer techniques and information on lymphoid leukosis and the rapid progress in the study of Marek's disease soon out-dated the 1964 edition; hence, the present second revision is necessary. The methods include those of many investigators in avian leukosis and cell culture as well as simplified and improved techniques developed at the RPRL.

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PROCEDURES FOR THE IN VITRO ASSAY OF VIRUSES AND ANTIBODY OF AVIAN LYMPHOID LEUKOSIS AND MAREK'S DISEASE

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INTRODUCTION

Avian leukosis remains one of the most important and costly problems in the poultry industry today; and, although much progress has been made in understanding the disease or diseases, effective control or prevention is still not available. Losses in the United States alone amount to well over \$150 million annually, and the disease is rampant in other countries. Therefore, effective methods for studying the causative agent or agents and immunological responses are vital, not only because of the poultry industry but also because of possible applications in other areas of cancer research. In vitro techniques have rapidly become of the utmost importance in the study of the avian leukosis complex, particularly since the advent of the RIF test in 1960.

The RIF test was first described by Rubin (33)¹ who demonstrated the presence of a transmissible agent in chick embryo fibroblasts (CEF) and in the growth medium of some CEF cultures that would induce resistance to neoplastic transformation of cells superinfected with Rous sarcoma virus (RSV). This agent was named RIF, an acronym for "resistance inducing factor." Subsequent investigation showed that RIF activity was characteristic of many viruses of the avian leukosis complex² (13, 33, 34, 37, 44). Soon afterward this characteristic became one of the important criteria for classification of diseases in this complex.

¹Italic numbers in parentheses refer to Literature Cited, p 16.

²BURMESTER, B. R., FREDRICKSON, T. N., and SOLOMON, J. J. Unpublished data. 1963.

These are currently classified into two groups, lymphoid leukosis (LL) for lymphoid proliferations caused by RIF positive viruses and Marek's disease (MD) for lesions of the nervous system caused by RIF negative agents (6).

The RIF test was followed in a short time by several other important developments, most of which will be mentioned only briefly. Perhaps the most significant was the discovery of a Rous associated virus (RAV) in 1962(34), since this paved the way for many subsequent important contributions. These include the discovery in 1965 by Vogt and Ishizaki (41, 42) and Hanafusa (15) that the RIF positive viruses occur in antigenically distinct subgroups, currently believed to be four (11); the finding that susceptibility to LL infection is determined by single autosomal genes specific for each subgroup (10); the observations on the defectiveness of RSV (16) and the production of non-RSV-producing cells (NP cells) (16, 39); and the role of helper lymphoid leukosis viruses in the maturation of RSV (16, 17, 40).

The observation that tumors could be induced in hamsters by RSV (1, 18) was also an important contribution for in vitro analysis of lymphoid leukosis virus (LLV), since it led to the development of a complement fixation test for avian leukosis (35) known by its acronym COFAL. This procedure was found to be as effective as the RIF test³ (14, 29) and less stringent in cultural requirements.

³SOLOMON, J. J., and OKAZAKI, W. Unpublished data. 1966.

Early in the 1960's Marek's disease was recognized as a major cause of leukosis mortality or condemnation in the poultry industry; hence, research emphasis rapidly shifted to this disease. These efforts culminated a few years later in the simultaneous development in two widely separated laboratories of cell culture assays and the identification of the agent as a member of the herpes virus group (9, 24, 38). As a result of these and subsequent findings,

prognosis for the control of MD appears better now than at any previous time. It is hoped that this publication on in vitro methods used at the Regional Poultry Research Laboratory, U. S. Department of Agriculture, and elsewhere will assist in the continued research on the avian leukosis complex and aid in its eventual control. It is not meant to serve as a comprehensive review or an all-inclusive publication on techniques.

CELL-CULTURE EQUIPMENT AND TECHNIQUES

(The superscripts (a-s) refer to sources for the equipment or media in the Appendix, p. 18.)

Equipment

Reusable Glassware

Glassware normally found in microbiology or tissue-culture laboratories are needed. High-use items include screwcap erlenmeyer flasks, large-volume (25- and 50-ml.) serological pipettes, filtration flasks with bottom takeoff outlets, aseptic filling bells, large aspirator bottles, and graduated cylindrical bottles.^a

Disposable Glass and Plastic

Tissue-culture plastic petri dishes^b of three sizes are used routinely. The 150- by 25-mm. size is used primarily for growth of primary cells, the 100- by 20-mm. size for passage of cells, and the 60- by 15-mm. size for assay of virus. Smaller sizes are also available and are occasionally used for special purposes. The special tissue culture dishes must be used. Disposable glass prescription bottles,^c 16- and 32-ounce, are used with or without washing for media storage. Disposable glass^a and plastic^b pipettes and syringes^d are also available and extensively used. The cost of the disposable items is probably less than the cost of washing and replacing expensive reusable glassware. Vacuum-formed plastic containers^e are useful as substitutes for glass beakers and for many miscellaneous uses.

Incubators

CO₂ incubators^f are used when cells are grown in petri dishes. A concentration of 3 to 5 percent CO₂ in air is desirable for most studies, and this can be obtained with flow meters. An alternate procedure is to adjust the CO₂ concentration until a pH of 7.0 to 7.4 is obtained in sterile media placed in the incubator. Too little CO₂ gives poor growth; whereas, too high a concentration is detrimental to the cells and may prevent the formation of RSV foci in infected cells. CO₂ is obtained commercially in cylinders and is mixed with air supplied by a small compressor^g equipped with a storage tank. The incubators may be used dry, but a high humidity is preferred to reduce the evaporation of nutrient fluid. This may be attained by adding water to the interior chamber of the incubator. Water-jacketed incubators maintain the temperature inside the incubator with less fluctuation and variation.

Microscopes

An inverted microscope^h is used to count RSV foci and MD microplaques in petri dishes and for general observation of cell monolayers. Magnifications most frequently used are between 25 and 50 \times . A 25 \times magnification is used for enumerating foci or microplaques, and the 50 \times is used for study of finer cell details. For these magnifications the most useful combinations are a 10 \times ocular with a 2.5 or 5.0 \times objective. A regular laboratory light microscope is used for counting cells in the hemacytometer.

Miscellaneous

Other equipment usually found in microbiology laboratories, such as an autoclave, sterilizing oven, stirrers, mixers, and hoods for aseptic procedures, is necessary. The hoods can be fabricated locally. The vortex type mixers are especially useful for dispersing cells.

Media Preparation—Stock Solutions

Medium 199.ⁱ—10×, Morgans, with L-glutamine, without NaHCO₃.

Nutrient mixture F10.ⁱ—10×, Ham's, with L-glutamine, without NaHCO₃.

BME basal medium.ⁱ—10×, Eagle, with L-glutamine, without NaHCO₃.

Tryptose phosphate broth (TPB), dehydrated powder.^j—The medium is rehydrated by dissolving 29.5 g. in 1,000 ml. water, and then dispensed in aliquots in 250-ml. erlenmeyer flasks or screwcap bottles. Then the flasks are sterilized in the autoclave for 15 minutes at 15 p.s.i.

Calf serum.—Purchased commercially.^{i k} Calf serum usually does not contain avian leukosis viral inhibitory substances when used at levels less than 10 percent. However, routine screening of calf serum before use is recommended, and all batches are tested for anti-leukosis-sarcoma virus activity and cell toxicity factors. Fetal calf serum is known to be inhibitory to RVS focus formation as is a high concentration (over 10 percent) of ordinary calf serum (32). Fetal serum has also been found to inhibit MD induced CPE in duck embryo cells. Newborn calf has been found to be satisfactory.

Calf serum received unfrozen is immediately inactivated at 56° C. for 30 minutes, then dispensed in about 100-ml. quantities, and refrozen until used. Serum received frozen is not thawed until used, at which time it is also inactivated as above.

Chicken serum.—Chicken serum when used as a nutrient or growth factor in cell culture should be free of LLV and RSV antibody, although there is some evidence that antibody may only mask virus without eliminating viral production by the cells (3). Most of the commercial chicken sera have been found to contain RSV antibody. Thus, RSV antibody-free serum

must be obtained from uninfected stock, and the following procedures are recommended for collection and processing of serum. The birds are exsanguinated by bleeding from the heart under negative pressure and the whole blood of three to five birds is pooled in a large sterile prescription bottle. The blood is allowed to clot at room temperature for about an hour. The clot is loosened from the walls of the container and subsequently refrigerated overnight. The next morning the serum is decanted and clarified by centrifugation for 15 minutes at 2,500 r.p.m. It is then drawn off and two to three lots pooled together and refrigerated. As soon as possible the individual pools are tested for RSV antibody using the different subgroup viruses.

Antibody negative pools are combined, passed through a sterilizing filter, dispensed in small aliquots in 100-ml. prescription bottles, and inactivated at 56° C. for 30 minutes. They are then tested for sterility and frozen until used. Filtration should be done through a filter with a pore size of 0.2 μ or less (Seitz S3 or Millipore GS filter), since *Pseudomonas* or other contaminants of small size are known to occur in blood or trypsin and may pass through filters of larger pore diameter.

Some commercially produced agamma chicken sera have also been found to be free of RSV inhibitory factors; such sera can be used when supplies of antibody-free sera are not available.

For use in Marek's disease studies, the same or even more stringent precautions need be taken to obtain antibody-free serum. Because of the rapid spread of the disease at an early age strict isolation procedures are required to produce infectious-free stock. Agamma sera have not been tested, but presumably they are as useful as in the studies on LL.

Sodium bicarbonate.—Twenty-eight grams of sodium bicarbonate (NaHCO₃), anhydrous, are dissolved in 1 l. of water, and the liquid is dispensed into flasks or bottles. The solution is then sterilized by autoclaving at 15 p.s.i. for 15 minutes. Water used in any cell-culture medium or reagent should be as highly purified as reasonably possible. A satisfactory procedure is to soften the water, distill it, and finally deionize it through a mixed bed resin.^l

Antibiotics.—A penicillin-streptomycin mixture is presently (1971) being used at a concentration of approximately 100 units and micrograms, respectively, per milliliter of final culture medium. The stock solution is prepared as a thousandfold concentrate, 1 ml. of which when added to 1 l. of culture medium gives the final concentration per milliliter. One vial of penicillin G potassium (2×10^7 units) and four vials of streptomycin sulfate (5 g./vial) are used to prepare 200 ml. of stock solution. About 5 ml. of sterile water is injected aseptically into each vial and the vials shaken until the antibiotics dissolve. The vial contents are then removed with a needle and syringe and brought up to a volume of 200 ml. with sterile water. The solution is then filtered through a Seitz S1 pad and dispensed in 5 to 10 ml. amounts in screw-cap vials that are stored frozen until used. (Filtration is an optional procedure utilized to help insure sterility.)

Other antibiotics have been used periodically for more specific control of contamination. These include the following with their respective stock concentrations.

Mycostatin—mold and yeast control	5,000 units/ml.
Amphotericin B—yeast control	500 units/ml.
Polymyxin—control of gram negative bacilli	2,000 units/ml.
Terramycin—control of pleuropneumonia-like organisms (PPLO)	5,000 units/ml.

Final working concentrations are a 1:100 dilution in the final culture medium.

Phosphate buffered saline base.—50× concentrate, less NaCl.

KCl	10 g.
KH ₂ PO ₄	10 g.
Na ₂ HPO ₄	60 g.
H ₂ O	1,000 ml.

All ingredients are dissolved in the water, dispensed in multiples of 20 ml., and stored frozen.

Trypsin.—Stock solutions of both 1.0 and 2.5 percent are used and are prepared as follows.

Either 10 g. (for 1.0 percent) or 25 g. (for 2.5 percent) of trypsin 1-300^m are added to 1 l. of phosphate buffered saline (reagent A, p. 5). The mixture is stirred overnight at 4° C. on a magnetic stirrer and then filtered through 1 to 2.5 cm. of celite filter aid.ⁿ The solution is then

final filtered for sterility through an S-3 Seitz pad (excessive foaming should be avoided), and the filtrate is dispensed in 5 to 10 ml. aliquots and frozen. Trypsin is sometimes variable in its activity; therefore, if desired, assays of trypsin activity for each new preparation can be done. Reference is made to Erlanger and coworkers (12) for details.

Agar.—Difco purified agar^j or Consolidated Laboratories ionagar #2^o have been used with success. Concentrations of 1.8, 3.0, or 3.6 percent are prepared by adding 18, 30, or 36 g., respectively, of agar to 1 l. of water. The mixture is boiled with constant stirring or heated in an autoclave for 10 minutes to dissolve the agar; the solution is then autoclaved at 15 p.s.i. for 15 minutes. The agar is melted in a boiling water bath just before use.

Working Media Preparation and Solutions

The media preparations used in this laboratory are listed below with the amount and type of stock solutions needed to prepare small volumes of 100 to 500 ml. and larger volumes of 1 to 6 liters.

<i>Stock solution</i>	<i>For volumes of 100-500 ml.</i>	<i>For volumes of 1-6 l.</i>
	<i>Milliliters</i>	<i>Milliliters</i>

Medium A.¹—Base medium for chick embryo fibroblast (CEF) and duck embryo fibroblast (DEF) growth and maintenance.

F10 (10X) ²	10.0	300.0
199 (10X) ²	8.0	240.0
TPB	10.0	300.0
Pen-Strept	.2	6.0
Mycostatin	1.0	30.0
Water	162.0	4,860.0

Medium B.¹—Base medium for chick kidney cell growth and maintenance.

BME (10X)	40.0	500.0
TPB	49.6	620.0
Pen-Strept	1.0	12.5
Mycostatin	2.6	32.0
Water	360.0	4,500.0

Medium C.¹—Hard agar base, NP test.

F10 (10X)	16.0	160.0
199 (10X)	12.5	125.0
TPB	17.5	175.0
Pen-Strept	.3	3.0
Mycostatin	1.5	15.0
Water	114.0	1,140.0

Medium D.¹—Hard agar base, RSV assay.

F10 (10X)	5.0	100.0
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Stock solution

For volumes of 1-6 l.
of 100-500 ml. Milliliters
Milliliters For volumes

Cell Preparations

Preparation of Chick Embryo
Primary Cells

Medium D—Con.		
199 (10X).....	5.0	100.0
TPB.....	14.0	280.0
Pen-Strept.....	.1	2.0
Mycostatin.....	.6	12.0
Water.....	40.0	800.0
Medium E.—Growth medium for CEF and DEF.		
Medium A.....	100	2,000
Calf serum.....	4	80
2.8 percent bicarbonate.....	4	80
Medium F.—Hard agar overlay, NP test.		
Medium C.....	60	600
3.6 percent agar.....	40	400
Bovine amniotic fluid.....	20	200
Calf serum.....	2.4	24
2.8 percent bicarbonate.....	5.0	50
Medium G.—Hard agar overlay, RSV assay.		
Medium D.....	60	600
1.8 percent agar.....	40	400
Calf serum.....	5	50
2.8 percent bicarbonate.....	4	40
Medium H.—Feed medium for agar overlay plates.		
199 (10X).....	10.0	100.0
2.8 percent bicarbonate.....	2.0	20.0
Pen-Strept.....	.1	1.0
Mycostatin.....	.5	5.0
Water.....	90.0	900.0
Reagent A. ³ —Phosphate buffered saline (PBS)		
PBS base (50X).....	20.0	60.0
NaCl.....	48.0	424.0
Water.....	980.0	2,940.0
Reagent B.—Trypsin (0.05 percent) for monolayer dispersion.		
Trypsin (2.5 percent).....	1.0	10.0
Reagent A (PBS).....	49.0	490.0
Reagent C.—Trypsin (0.125 percent) ⁵ for embryo cell dispersion.		
Trypsin (1.0 percent).....	10.0	100.0
Reagent A (PBS).....	70.0	700.0

¹ Various concentrations of NaHCO₃, bovine serum, and other additives are added in the different tests. These are indicated later.

² CEF have been found to grow better in a mixture of medium 199 and medium F10; also focus formation by RSV was better and the titer higher in this medium (unpublished data of J. J. Solomon and K. Glatt). It has therefore been adopted for most fibroblastic cultures in our Laboratory.

³ The 50× concentration is thawed and added with sodium chloride to the water. The ingredients are thoroughly mixed and dissolved, and the solution is dispensed in bottles. The solution is autoclaved for 15 minutes at p.s.i. The pH should be between 7.0 and 7.4.

⁴ Grams instead of milliliters.

⁵ Trypsin at 0.25 percent is also satisfactory for embryo cell dispersion. It is prepared by diluting either the 1.0 or 2.5 percent trypsin.

Chick embryo primary cells are prepared in the following manner. The outside of the shell of a 9- to 11-day-old embryonated egg is disinfected by immersion in 70 percent ethyl alcohol for 5 minutes. The shell above the air cell is then cut away with scissors, and the embryo is lifted out of the egg, decapitated, and placed into a 125- or 250-ml. erlenmeyer trypsinization flask containing about 30 ml. of sterile PBS (reagent A). Usually 3 to 5 embryos from the same dam are pooled in one flask. The embryos are washed at room temperature on a magnetic stirrer for approximately 3 minutes. This is repeated with fresh PBS until the saline remains fairly clear. The embryos are then macerated by holding the flask in a tilted position and allowing the stirring bar to rotate at a moderately fast speed so that it forces the embryo against the flask. This operation requires about 1 to 3 minutes per flask. It avoids opening the flask for mincing the embryos with scissors or spatula and thus decreases the chance for contamination. In general, the smaller the tissue pieces, the greater the final cell yield. The macerated tissue is washed for 2 or 3 minutes, the tissue fragments are allowed to settle, and the supernatant saline is removed. The procedure is repeated until the saline remains fairly clear. After the final wash, about 100 ml. of warm trypsin (37° to 48° C.) (reagent C) are added to each flask, and the flask contents are stirred slowly on a magnetic stirrer for 30 to 60 minutes. For optimum trypsinization the temperature should be at 37° to 48° C. during this period. The heavier particles are allowed to settle for 1 to 3 minutes and supernate is removed and centrifuged at 500 r.p.m. for 15 minutes or 1,200 r.p.m. for 3 minutes. The cell pellet is resuspended in medium E and mixed by aspirating or with a vortex mixer, and cells are counted⁴—care should be taken not to include the nucleated red blood cells. If the cell suspension is heavy (greater than 3 to 5 × 10⁶ cells/ml.), it should be diluted (1:5 to 1:10)

⁴ For use of hemacytometer, see Appendix, p. 18.

and recounted. The cells are then plated in medium E at concentrations of 1×10^7 cells in 8 to 10 ml. for 100-mm.-diameter Falcon plastic tissue-culture dishes or 2.0×10^7 cells in 20 to 25 ml. for 150-mm.-diameter dishes. The latter size is more efficient in cost and labor.

Cells of known RSV sensitivity phenotype must be used in studies on lymphoid leukemia. Sensitivity is determined by the following procedure. One million primary cells are seeded in 4 ml. of medium E in 60-mm.-diameter petri dishes, and the plates are incubated overnight at 37° C. The medium is removed and replaced with 4 ml. of fresh medium to eliminate cells that did not adhere and grow. The cells are then challenged with 0.1 ml. of RSV from each subgroup, diluted in medium A to contain 10^3 and 10^4 focus-forming units (FFU) of virus. Two plates are challenged with the low dose and two with the high dose from each subgroup, and 2 to 18 hours later the liquid medium is removed and 5 ml. of agar overlay (medium G) added. The plates are reincubated for 3 days and 2 to 3 ml. of feed medium H added over the agar. The cells are observed 4 to 7 days after infection for the presence of mature foci and a count of foci is made during this period. (For details on development and counting of RSV foci, see p. 7.)

With the high dose of virus, sensitive cells will show signs of RSV infection within 3 or 4 days. These appear as high acidity compared with noninfected controls and the presence of RSV foci. Some evidence of embryo sensitivity can thus be rapidly obtained from the high-dose challenges and sensitivity primary cells selected for experimental purposes shortly thereafter. The plates challenged with 1,000 FFU should be held 4 to 7 days before the foci are counted. This gives quantitation of the cell sensitivity to RSV. Cells showing a RSV focus count ≤ 0.1 of the infecting dose are considered to be resistant to RSV, owing to the presence of LL viruses or inherent genetic factors. Genetic resistance can be distinguished from viral resistance by lack of transmission with cell-free fluids from the monolayer.

Preparation of Duck Embryo Primary Cells

Duck embryo fibroblasts are used in the study of Marek's disease and in the NP test for LL viruses. They are prepared in the same manner as the chick embryo primary cells with the exception that 13- or 14-day-old embryos are used. Cells from all sources tested have been sensitive to the herpes virus of MD; thus primary-cell sensitivity tests are not necessary.

Preparation of Chick Kidney Cells

Chick kidney cells are also used in the study of MD. They can be obtained from embryos, young chickens (1-3 weeks old), or adult birds. The general procedure is to remove the kidneys aseptically, wash them with PBS in a small beaker, and mince them with scissors. An alternate procedure is to inject heparin intravenously just before the chicken is killed and immediately after to perfuse the kidney with 0.05 percent trypsin via the common iliac vein. The minced kidney is then trypsinized for two cycles of 5 to 10 minutes with 0.05 percent trypsin, and the dispersed cells are discarded. Several additional trypsinization cycles are carried out, and the cells are collected and plated in medium B containing 0.084 to 0.112 percent bicarbonate (3 to 4 ml. 2.8 percent/100 ml.) and 5 percent fetal bovine serum. The serum content is reduced to 2 percent after a confluent layer is obtained, 1 to 3 days after plating.

For tissue-culture procedures in general, the textbook by Parker (25) and the laboratory manual by Merchant and coworkers (22) are recommended. In our Laboratory we have found that changing growth medium on the monolayers on alternate days gives better cell growth and maintenance. It is also very important to maintain the pH near neutrality and this often requires a neutralization of the acid produced by the cells on the day after the medium change. This is done by the addition of enough (0.1 to 0.5 ml.) sterile 2.8 percent

NaHCO₃ to the plates to bring the color back to that of fresh medium. It is essential that cells be observed frequently and that overcrowding be avoided by cell transfer or the use of maintenance medium after confluent monolayers are

achieved. For maintenance purposes, the serum content in the growth medium can be reduced to 1 to 2 percent or 10 percent bovine amniotic fluid can be added to the regular media formulations.

VIRUS PREPARATIONS

Rous Sarcoma Virus (RSV)

Tumor Induction and Harvest

Stocks of standard RSV of high potency may be propagated in cell culture *in vitro* or extracted from tumors induced in chickens *in vivo*. Stocks of virus with titers of approximately 10⁶ to 10⁷ FFU per milliliter are readily obtained by either method. For the *in vitro* propagation of RSV, Bader's procedures (2) are recommended. The following is a brief description of the induction of tumors in chickens and the subsequent extraction of virus by Moloney's method (23).

Susceptible chickens 3 to 5 weeks of age, free of LLV, are inoculated intramuscularly in the pectoral muscle or subcutaneously in the wing web with 0.2 ml. of RSV containing 10³ or 10⁴ infectious units of virus. Beginning at 5 days after inoculation, the chicks are examined daily for tumor growth at the site of inoculation. The growth of the tumors is very rapid; the tumor tissue should be harvestable between the seventh and 14th day. During this time the pectoral region of chicks inoculated at this site will enlarge to several times its normal size, and tumors induced in the wing web should approach 15 to 20 mm. in diameter or larger. The tumor growth should not be allowed to progress to the hemorrhagic, necrotic, or ulcerative state, which will occur if held too long. The tumor masses should be harvested using aseptic techniques; care should be taken not to include hemorrhagic and or necrotic areas. The excised tumor material should be kept cold, preferably in containers on ice. It is pooled, minced, frozen, and stored at -70° C. as soon as possible after harvest.

Partial Purification (Moloney's method (23)): The frozen tumor mince is thawed at room temperature and made up to 6.6 percent (wt./v.) with 0.15M potassium citrate containing 1 mg. percent hyaluronidase. The suspension is allowed to digest at room temperature in a Waring Blender with intermittent mixing (15 seconds at 10-minute intervals) for 1 hour. At the end of this period, it is homogenized thoroughly for 3 minutes. The homogenate is centrifuged in the cold at 2,300 × g. for 20 minutes, and the supernatant fluid is carefully pipetted off; the top fatty and lower sediment layers should be avoided. This is designated the S₁ fraction. The centrifugation (2,300 × g. for 20 minutes) is repeated, and the resultant fluid is designated S₂. The S₂ fraction is centrifuged at 18,000 × g. for 1 minute; the supernatant (S₃) is poured off and retained. The S₃ fraction is passed through a bacteriological filter, vialled, sealed, and stored at -70° C.

Assay of RSV

The first step is to obtain RSV sensitive primary cells. This can be determined from the results of the primary challenge or from a past history of RSV sensitivity of embryos from a particular dam. However, the sensitivity of the cells must also be confirmed during the particular experiment in progress. The CEF monolayers are prepared by decanting the fluid layer from the RSV sensitive primary cells into a container fitted with a gauze cover to reduce splash. Six to ten ml. of warm (37° C.) 0.05 percent trypsin are added to each plate, and the plates are reincubated at 37° C. for 10 minutes. This procedure usually loosens most

of the cells. The remaining attached cells can be removed by forcing the trypsin-cell mixture against the bottom of the petri dish through a 10 ml. pipette or by scraping with a sterile rubber policeman. When all cells have been removed, the suspension is transferred to a large centrifuge tube. The cells from several plates are pooled together in the tube and centrifuged at 1,200 r.p.m. for 3 minutes. They are then resuspended in medium E (about 7 to 10 ml. for each plate of cells added to the pool) and counted in a hemacytometer. One million cells are then added to a small petri dish, and the volume is brought up to 4 or 5 ml. with medium E. The plates are gently shaken until the cells are evenly distributed and then placed in the incubator.

The virus is now diluted with feed or growth medium—serial tenfold dilutions should be used. All manipulations of the virus before addition to the CEF cultures should be done in the cold. One-tenth ml. of each dilution of virus is then added to each of two petri dishes prepared above, and the plate contents gently but thoroughly mixed. Next morning the fluid medium is removed and replaced with 5 ml. of agar overlay medium G. The interval between infecting the cells and overlaying the agar should not exceed 18 hours, since virus may then be produced by the cells and the titer will appear to be higher. Four days after infection, 2 or 3 ml. of feed medium H are added to each plate.

Cells infected with RSV show no visible change during the first 2 days. On the third day they become granular and also appear darkened. Clusters of rounded up cells can be seen throughout the plate by the fourth day, and mature foci of rounded up cells develop shortly thereafter (fifth day). The sixth and seventh day cells show the foci clearly. Development of foci is asynchronous; therefore, foci usually should be counted as late as 7 days after infection. With some virus strains and under certain culture conditions, the whole cycle of focus development is shortened and counting can be done by the fourth or fifth day. Some cells are more sensitive to the virus than others; hence, careful observation of the plates is necessary since sensitive cells are rapidly destroyed by high concentrations of virus.

Counting of the foci is done with an inverted microscope. The center piece on the stage is removed and a counting grid¹⁹ installed in its place. The foci on a representative fraction of the area of the plate are counted, and the total foci per plate are calculated by multiplying this number by the reciprocal of the area counted. The procedure is valid only when uniform distribution of the foci are obtained. Where only a few foci are present, the foci on the entire plate are counted. Since each focus is presumably initiated by a single infectious unit, a count of foci gives a titer of the RSV preparation. However, Rubin (31) has suggested the addition of 1 log to the titer, since he found that of 10 infected cells only one actually goes on to initiate a focus.

Lymphoid Leukosis Virus (LLV)

Propagation.—Strain RPL-12, subgroup A virus has frequently been used in the study of lymphoid leukosis and related neoplasms in vivo and as an RSV-resistance-inducing virus in tissue culture. Virus propagation at this Laboratory has primarily been done in vivo (5). For tissue-culture procedures of propagation of LL virus, the papers by Rubin (33) and Bader (2) are recommended.

End point assay.—The end point assay of LL virus is done by the RIF procedure. This is detailed in the section on virus assay, lymphoid leukosis, under RIF test.

Marek's Disease (MD)

Propagation of the agent has been done primarily in vivo because of the highly cell-associated nature of the agent and the absence until recently (9, 38) of in vitro techniques. Details on the in vivo propagation of two strains can be obtained by reference to Witter and coworkers (45).

In vitro propagation can be done on either chick kidney or duck embryo cells. The latter procedure will be described in detail. DEF primary cells are trypsinized in the same manner as CEF primary cells in the RSV assay. The cells are then counted and plated at 3×10^6 cells per 100-mm. petri dish in medium E and

incubated overnight. One or more plates each are then inoculated with 0.2 ml. of tissue infected with the agent to be propagated. Whole blood, buffy coat, tumor, or other tissue can be used. The plates are reincubated for 1 to 24 hours, the inoculum gently washed off with PBS, and the plates maintained under liquid for up to 12 days. Herpes virus plaques began to appear between the fifth and eighth day after infection, and the cells can be harvested shortly thereafter. The cells are removed by trypsinization, resuspended in medium A containing 15 percent calf serum and 5 percent DMSO, and vialled in 1-ml. quantities at a concentration of

5 to 10×10^6 /ml. Cells in DMSO must be kept on ice. The vials are then sealed, the temperature immediately lowered at a rate of about 1°C./minute down to -40° , and the vials stored in liquid nitrogen.

Assay of the agent is done by titrating the infected cells on DEF monolayers in the same manner as in the propagation of the agent. Plaque morphology is described under Marek's disease in the section on virus assay. Increases in titer can be obtained by repeating the propagation procedure with the infected DEF as the inoculum.

VIRUS ASSAY

Lymphoid Leukosis

There are a number of in vitro assay procedures for LL virus, but only the more commonly used or newer techniques will be described in detail. These include the RIF, COFAL, and NP activation procedures. The latter is the result of work by many investigators but in particular the work of Hanafusa and others (15, 16, 40) on the defectiveness of RSV and the helper function of leukosis viruses. Rispen and coworkers (30) established an in vitro system for this assay procedure by cocultivating NP CEF with duck embryo cells. The addition of test material containing LL virus to this system activates the NP cells to produce RSV. The presence of RSV in the cell-free culture fluids can then be detected by one of several techniques to confirm the presence of LL virus in the test sample. The NP activation test is rapid and simple after the preparation of NP cells and compares in sensitivity to the RIF and COFAL assays (30). Information on other procedures for the assay of LL virus can be found in the references cited or in other literature. For information on the use of fluorescent staining techniques in studies on LL virus (7, 19, 20, 21, 26, 43) are suggested.

Resistance Inducing Factor (RIF) Test

The RIF test can be initiated on either primary or secondary RSV sensitive CEF. Cells are

plated at a concentration of 5 to 10×10^6 primary or 2 to 3×10^6 secondary cells in 100-mm. petri dishes, and 0.1 ml. of test material (serum, plasma, tumor extract, or similar material) is added to the plates shortly thereafter (up to 18 hours after plating). Duplicate plates should be inoculated with each sample and positive and negative controls included in each test. Negative controls are of two types, i.e., plates that are uninoculated and plates inoculated with known LL virus-free chicken serum. Positive controls are inoculated with serial dilutions of standard LL virus of all known subgroups or with dilutions known to encompass the endpoint titer of the standard viruses.

The plates are incubated for 3 days, the fluid medium is discarded, and the cells are removed by trypsinization. After centrifugation the cells are resuspended in growth medium E and counted. One million cells are then added to each of four small assay plates (60-mm. diameter), and 2 to 3×10^6 cells added to one or more 100-mm. plates. The larger plates are immediately reincubated for 3 or 4 days for use in the second transfer (see below). Pseudotype RSV from each subgroup is diluted to contain about 10^4 FFU/ml. and 0.1 ml. is used to challenge the small plates, one subgroup per plate. On the following day, the fluid medium is removed and replaced with agar overly medium G. Three days later, 3 ml. of feed medium H

are added to each plate, and foci are counted 4 to 7 days after infection. This constitutes the first transfer and first RSV challenge.

Three or four days later the second transfer and challenge are made with the large plate(s) of cells from the first transfer in the same manner as with the originally infected large plate, i.e., trypsinize, count, and seed the cells into four small plates for subsequent RSV challenge and into one or more large plates for the next transfer. This procedure is continued until no further increase in resistance is found with the higher dilutions of control LL virus. Two to four transfers are usually adequate to detect most virus in test materials, but several additional transfers are desirable and should be conducted if the condition of the cells permits. Challenge with RSV may be restricted to alternate transfers. A reduction ≥ 1 log (≥ 90 percent) in the number of foci on test plates compared with the negative controls is positive for the presence of LL virus.

The titer of an unknown can be estimated by reference to a standard LL virus titration curve (endpoint assay of LL virus), which is done in the following manner: RSV sensitive CEF are prepared as in the RIF test and inoculated with serial tenfold dilutions of standard LL virus. The cells are passaged at 3-day intervals, as in the RIF test, and challenged at each passage with RSV from the same subgroup as the LL standard virus. The procedure is continued until no further increase in resistance is found with the higher dilutions of virus. The endpoint is usually obtained after 2 to 4 transfers, but this must be determined for each strain of virus. The data are analyzed by plotting relative sensitivity (RSV focus count of sample/RSV focus count of negative control) against log virus dilution for each passage (fig. 1). The titer of the standard virus can then be determined from the graph. In the figure shown a titer of 10^7 infectious units/0.1 ml. (10^8 /ml.) is obtained, and in this instance the endpoint is reached after only two passages.

From a standard LL virus curve, such as that shown in figure 1, the titer of an unknown can then be estimated. For example, the titer of a sample tested undiluted and inducing ≥ 1 log resistance on the first challenge would be be-

tween 10^6 and 10^8 infectious units per milliliter. Failure to obtain resistance on the first challenge would indicate a titer at least 4 logs less than the standard LL virus if resistance was obtained on the second and third challenge. The comparative interference assay of Vogt and Rubin (44) can also be used to determine the titer of an unknown LL virus. In this procedure the titer of a standard virus is first determined by the endpoint assay (as above). A sample of the standard and the unknown are then diluted serially and used to inoculate different chick embryo cultures. The cells are transferred and challenged 3 days later, and the level of resistance of each dilution is determined. The relative sensitivity to RSV is then plotted as a function of the sample dilution on a logarithmic scale. Parallel straight lines are obtained, the curve of the unknown being displaced horizontally from that of the standard. This displacement corresponds to a dilution factor and the titer of the unknown is less than or greater than the titer of the known by this factor.

Complement Fixation for Avian Leukosis (COFAL) Test

The COFAL test is performed in two phases: (1) LL virus propagation or antigen production in CEF cells, and (2) assay of specific complement fixing antigen. In addition to the standard complement fixation (CF) test reagents obtainable from numerous commercial sources,^{1,2} the COFAL test requires the following: (1) LL virus susceptible chicken embryo fibroblasts; (2) stock COFAL positive antigen, which may be obtained as extracts from RSV induced chicken tumors, RSV or LLV infected CEF, or RSV induced hamster tumors; and (3) stock positive antiserum (hamster), which may be produced by the inoculation of newborn or weanling hamsters with RSV or RSV induced hamster tumor cells and the subsequent collection of serum from tumorous hamsters. However, since this procedure is time consuming and the titer and specificity of the serum from each hamster are highly variable, it is strongly suggested that antiserum be purchased from a commercial source.⁴

The test samples are inoculated onto primary or secondary CEF and held in culture for ap-

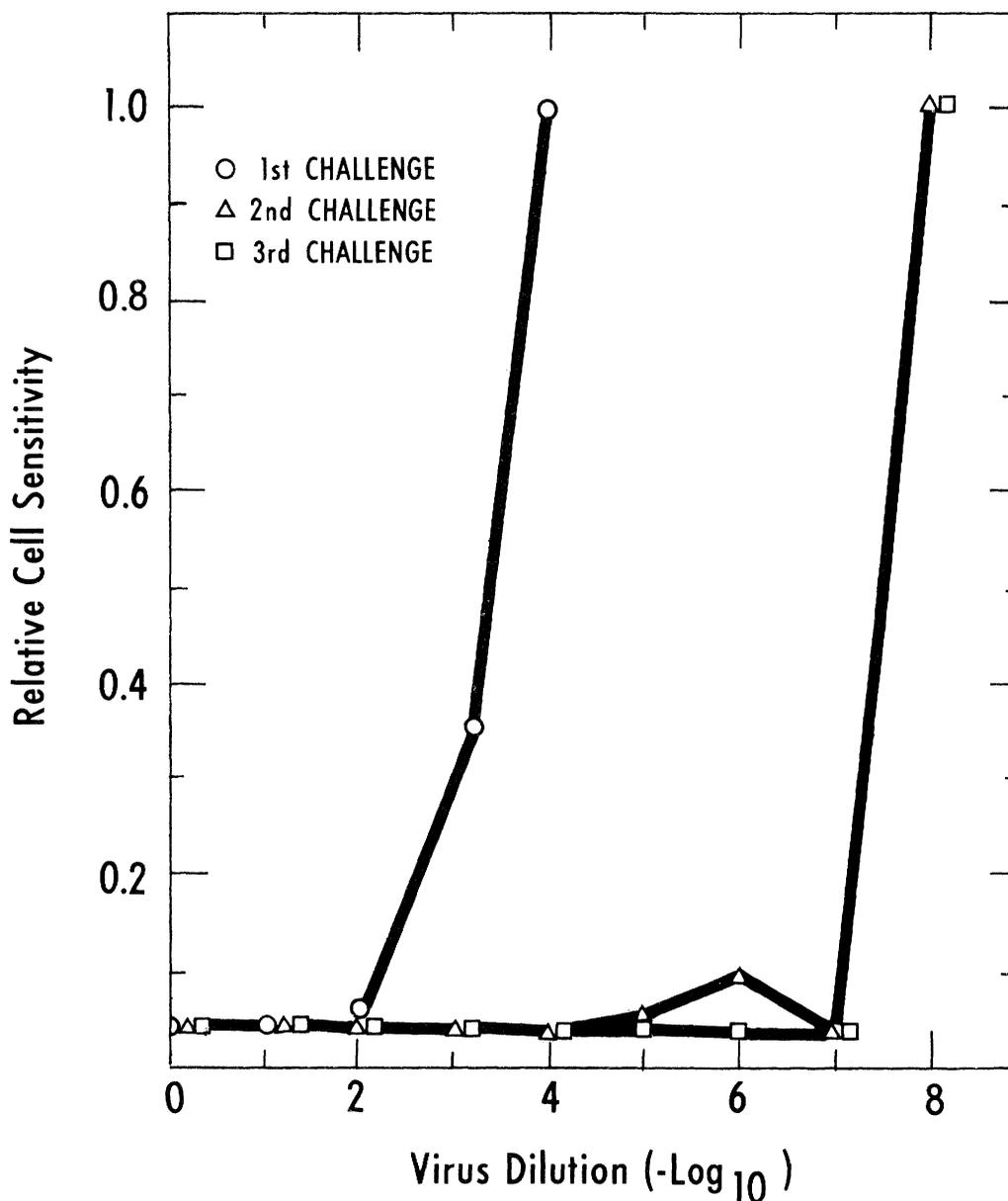


FIGURE 1.—RIF assay curve of RPL12—L31, Lymphoid leukemia virus.

proximately 21 days with cell transfers at 3- to 5-day intervals, as in the RIF test procedure. Positive controls, consisting of CEF inoculated with known LL viruses from the various subgroups, as well as uninoculated control CEF cells are required for each test. Although some of the rapidly propagating viruses each endpoint titers in 12 to 14 days, other slower growing types require somewhat longer. At the end

of the culture period, all but 0.2 to 0.3 ml. of the fluid is removed and the cells are scraped free of the culture surface with a rubber policeman and suspended in the retained fluid. The cell suspension is frozen and thawed three times (alcohol-dry ice) or sonicated to rupture the cells and then clarified by low-speed centrifugations; the clear fluid is used as antigen in the complement fixation test.

The microtiter^r system (36) is highly recommended for use in the complement fixation phase (4) of the COFAL test. It has been shown to be accurate, and highly reproducible results can be obtained (36). In addition, a small volume of reagent aliquots (0.25 ml.) is required and three to six sample dilutions can be made in one operation; hence, other standard CF methods appear obsolete.

The CF phase of the test is performed as follows: The test sample is diluted twofold across a series of wells. Anticomplementary (AC) controls are included as the two highest concentrations of test material. The 0.025-ml. loops and dropper pipettes are used for this dilution. Veronal buffered saline, pH 7.3, is used for all reagent diluent. Antiserum (hamster, 4 units in 0.025 ml.) is added to all test samples except the AC controls, in which veronal buffered saline is used. Complement^j (2 exact units in 0.025 ml.) is added to all wells, test samples as well as AC controls. The plates are gently shaken, (precautions must be taken not to splash the dilution over from well to well) and held static at 4° C. overnight (approximately 16–18 hours). The following morning, sheep erythrocytes^{i,j} collected or stored in Alsever's solution (4) are washed three times in veronal buffer, resuspended to a 2 percent concentration, and sensitized for 15 minutes at room temperature with an equal quantity of diluted hemolysin^j to contain 2 units per 0.025-ml. aliquot; 0.05 ml. of these sensitized sheep erythrocytes are added to each well. The plates are carefully sealed with wide transparent cellophane tape, shaken vigorously, and held at 37° for 1 hour with a shaking after the first 10 minutes of incubation. The plates are then held in a static position at room temperature for 1 hour and read for fixation. Fixation, or a positive reaction for LL antigen, is indicated by the absence of lysis of sheep erythrocytes. The following controls are always included with each test: (1) Standard positive antigen AC control, (2) standard positive antiserum AC control, (3) complement activity controls of three concentrations—2, 1, and 1/2 units, (4) erythrocyte

fragility control, and (5) known positive system control.

Nonproducing (NP) Cell Activation Test

The procedure and reagents found most suitable for the NP test are outlined below. Two phases are required for this procedure: (1) production of NP cells and (2) assay of LL virus.

(1) NP cell production

a. Materials

- (1) CEF as in RIF and COFAL procedures.
- (2) DEF
- (3) BH-RSV (Rav-1) or other BH-RSV pseudotypes.

b. Procedure

Primary or secondary cells are plated at a concentration of 1 to 2×10^4 CEF and 10^6 DEF in 60-mm. petri dishes. The cells are infected while in suspension with 0.2 ml. of serial twofold dilutions of RSV, duplicate plates for each dilution. Plates with 50 foci or less are desired for cloning, and appropriate dilutions of RSV to be used can be determined by titering the virus beforehand. After overnight incubation, the cells are overlaid with 5 ml. of agar overlay medium F containing high titer chicken antiserum prepared against the RSV being cloned. Five days later a second overlay of 2.5 ml. is added onto the previous agar layer. Ten to eleven days after inoculation, about 25 isolated, large dense foci without degenerate centers are selected and the agar immediately above is cut with a sterile cork borer and removed aseptically. Contact between foci must be avoided to prevent possible cross contamination with RSV. The cells are then trypsinized by adding a small amount of

1 percent trypsin (diluted 1:20 to 1:50 with PBS) and harvested with a pasteur pipette. The trypsin action is reduced by cooling or by the addition of about 0.5-ml. calf serum per focus harvested. The cells obtained from each focus are plated on freshly prepared secondary DEF, one focus per 60-mm. plate containing 1.0 to 1.2×10^6 DEF in medium E. The cells are then subcultured at 7- to 10-day intervals, and at each subculture supernatant fluid is tested for the presence of cell-free RSV by either in vitro cell assay or in vivo chorioallantoic membrane (CAM) assay. Only those clones free of infectious RSV are retained and used in the production of NP cells.

The in vitro assay for cell-free RSV is done by adding 2 to 4 ml. of culture fluid to RSV sensitive, RIF-free CEF, followed by an agar overlay 18 to 24 hours later. Three days after infection, 3 ml. of feed medium is added, and 3 or 4 days later the monolayer is observed for foci.

CAM assay is done by inoculating 0.2 ml. of culture fluid onto the CAMs of 9- or 10-day-old RIF-free, RSV-sensitive embryonated eggs. The CAMs are removed and examined for pocks 8 or 9 days later.

Cells that are found to be nonproducing are then propagated through several passages until the number of cells is adequate for large-quantity storage. At the start of each passage, fresh secondary DEF are mixed with the NP cells to obtain confluent foci at the end of the passage and increase the number of cells. The ratio is generally determined by visual inspection of the cell monolayer at intervals before passage. A 1:1 ratio of NP to fresh DEF cells has been found to be satisfactory during many of the passages. About five passages are required

to increase the number of cells sufficiently to obtain storage quantities. Medium E with 1 percent calf serum or 2 percent bovine fetal serum plus 10 percent bovine amniotic fluids (BAF) has been found to be an excellent maintenance medium for cells 24 hours after transfer.

At the end of the propagation period, the NP cells are harvested by trypsinization and resuspended at a concentration of 5 to 10×10^6 per ml. in medium E containing 20 percent serum, 10 percent BAF, and 5 percent DMSO. (Cells in DMSO should be kept in ice.) They are then vialled in 1-ml. quantities and frozen by lowering the temperature from 5° to -40° C. at the rate of 1° per minute. The sealed vials are then stored in liquid nitrogen.

- (2) Assay for LL virus (NP cell activation and RSV assay)

Direct Activation Test

- a. The NP cells are removed from liquid nitrogen storage and rapidly thawed in water at 37° C.
- b. Fresh secondary DEF, NP cells, and fresh secondary CEF (type C/O, LL virus free) are plated at 1.0 , 3.0 , and 4.0×10^5 , respectively, in a 60-mm.-diameter plate.
- c. These cells are inoculated 24 hours later with 0.2 to 1.0 ml. of test material per plate.
- d. Plates are incubated overnight at 37° C., the culture fluid is removed, and the cell monolayer is washed with PBS. The growth medium is replaced with maintenance medium (medium E, 1 percent calf or bovine fetal serum, and 10 percent BAF).
- e. The fluid is harvested on the fifth, seventh, or ninth day, and the RSV is assayed by either CEF or CAM assay.
- f. The following controls are required during this test procedure:

Component	Purpose
(1) Monolayer of NP cells alone. ¹	To assure the nonproducer status of the NP cells.
(2) Monolayer of NP and DEF cells. ¹	To test the DEF for congenital or adventitious infection with LL virus.
(3) Monolayer of NP and CEF cells. ¹	To test the CEF for congenital or adventitious infection with LL virus.
(4) Monolayers of NP cells infected with dilutions of LL virus of the known subgroups (span endpoint dilutions). ²	For positive control on the sensitivity of the NP system.

¹ Culture fluid from controls (1), (2), and (3) must not contain RSV.

² Culture fluid from control (4) should contain RSV through the endpoint LL virus dilutions.

The NP cell activation test can also be used to test for the presence of LL virus in cultured CEF. This procedure is frequently used in the RIF test to determine the infection status of the embryo. This is done by adding the CEF being tested to fresh secondary DEF and NP cells and assaying the culture fluid for cell-free RSV (as in steps d and e of the direct activation test.)

Marek's Disease

Marek's disease virus can be assayed in cell culture by the use of either duck embryo fibroblasts or chick kidney cells. The chick kidney system appears to be somewhat more sensitive than the duck cell system. When virus assays are to be conducted on chickens to be sacrificed, direct culture of the kidney is preferred because it is considered somewhat more sensitive.

For chickens that cannot be sacrificed, the following techniques are used.

Indirect kidney cell test.—Chick kidney cells are prepared and plated at a concentration of 5 to 8 × 10⁶ per 60-mm.-diameter plate in medium B with bicarbonate and serum, as indicated under preparation of kidney cells. After 24 to 48 hours the plates are inoculated with the material being tested (whole blood, tumor cells, tumor extracts) and reincubated. In 3 to 24

hours after inoculation, the inoculum is washed off with PBS and fresh medium is added. When the cells are confluent (usually 48–72 hours) the medium is changed to a maintenance medium by reducing the concentration of bovine fetal serum to 1 to 2 percent. The cytopathogenic effect (CPE) can be observed 3 to 5 days after inoculation and enumerated 9 to 10 days after inoculation. The CPE is easily distinguished and consists of rounded or elongated focal clusters of cells against a flat monolayer of normal cells. Positive and uninoculated controls are carried in each experiment.

Duck embryo cell assay.—The duck embryo cell assay system is similar to the kidney cell system; secondary DEF are inoculated with the material under test, and cells are observed 7 to 14 days after infection for CPE. Secondary cells are plated in medium E at 1.0 × 10⁶ per 60-mm.-diameter plate and inoculated up to 48 hours later, generally with 0.1 ml. of inoculum. Higher concentrations of some inocula may be toxic to the cells. In 3 to 24 hours after inoculation, the inoculum is washed off with PBS and fresh growth medium is added. CPE generally first appear in 5 to 7 days and may be enumerated 10 to 14 days after infection. During this period the plates must be observed for cell crowding and decreased pH. In some instances a maintenance-type medium, medium E with 1 to 3 percent calf serum, is substituted. It is important that the cell physiology be as optimal as

possible. The CPE that appears is similar to that obtained with the chick kidney cell monolayers except that there are fewer round cells and more elongated cells. Positive and negative

controls should be carried in each experiment. Bovine fetal serum has been found to be detrimental to the formation of CPE in the DEF system; hence, it should not be used.

ANTIBODY ASSAY

Lymphoid Leukosis

Antibody to LLV is measured by its reaction with RSV and the neutralization of RSV is determined by any of the RSV assay procedures, i.e., chick or embryonated egg inoculation and cell culture. Only the latter in vitro technique will be given in detail.

RSV of the subgroup(s) of interest are selected for the assay and diluted to contain about 10^4 FFU/ml. The test serum or plasma is inactivated at 56° C. for 30 minutes and 0.1 ml. incubated with 0.9 ml. of virus at 37° for 40 minutes. One-tenth ml. is then plated on sensitive CEF and overlaid with agar on the following day. The number of foci are counted 4 to 7 days after infection. A reduction in the number of foci of ≥ 90 percent is considered positive for Rous virus antibody. Negative sera fail to inhibit the virus and the number of foci is similar to that found on the control cells inoculated with virus alone. The antibody titer of the serum is expressed as the dilution of serum that reduces the titer of RSV by 1 log or more.

Marek's Disease

Two techniques, only recently available (8, 27, 28) have been used to detect antibody to MD—the agar gel precipitin test and the fluorescent staining technique.

Agar gel precipitin test.—The agar gel precipitin test of Chubb and Churchill (8), with either MD infected chick kidney cells or duck embryo fibroblasts as the antigen, is a rapid and simple technique for detecting antibody in sera or plasma. Antigen is prepared by infecting cells with MD agent and culturing the cells through several passages until a number of plates with confluent or heavy CPE are available. At this point the cells are trypsinized, resuspended in

PBS at a concentration of 4×10^7 /ml. and rapidly frozen and thawed three times. The antigen is then ready for testing against a known antiserum. This is done on an agar gel slide or plate by means of the Ouchterlony technique. Agar is melted, a preservative (either 0.5 to 1.0 percent phenol or 1:10,000 merthiolate) is added when the agar is about 45° to 48° C. and slides or plates are prepared. Small circular wells are then cut in the agar in a symmetrical pattern around a central well with a cork borer or patterned cutter and serum and antigen added to adjacent wells. The agar slide is observed at 24 and 72 hours for the formation of precipitin bands between serum and antigen, which indicates a positive reaction.

Fluorescent staining technique.—First the antigen is prepared by infecting 24- to 48-hour DEF or CK cells on coverslips with MD agent. The cells are cultured for 3 to 5 days after infection, fixed in acetone at 4° C., and air dried. The antigen thus prepared is ready for use or the cells can be stored frozen for future tests. The test for antibody is made by flooding serum or plasma diluted 1:20 in PBS onto the cells and keeping it at room temperature for 30 minutes. The cells are then washed in PBS for 15 minutes, flooded with anti-chicken conjugated globulin,^s and again incubated at room temperature for 30 minutes. The wash procedure with PBS is repeated, and the cells are rinsed with distilled water and mounted on a slide in glycerin buffer or some other nonfluorescing mounting medium. The slide is then observed for staining by fluorescent microscopy. The test correlates well with the agar gel precipitin test and appears to be more sensitive. For additional details on the procedure the references by Purchase (27, 28) are recommended.

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APPENDIX

Sources of Equipment and Media

- a. Bellco Glass, Inc., Vineland, N. J. 08360; Owens, Illinois (Kimble Products), Toledo, Ohio 43601; and Corning Glass (Pyrex brand), Corning, N. Y. 14832, are three of several good sources for tissue-culture glassware.
- b. Falcon Plastics, 5500 W. 83rd Street, Los Angeles, Calif. 90045.
- c. Brockway Glass, Inc., 11000 W. McNichols Road, Detroit, Mich. 48221.
- d. Becton, Dickinson and Company, Rutherford, N. J. 07070.
- e. General Food Container Company, 1016 East 11th Street Indianapolis, Ind. 46202.
- f. Labline Instruments, 3070-82 W. Grand Avenue, Chicago, Ill. 60622 and National Appliance Company, 10855 S. W. Greenburg Road, P. O. Box 23008, Portland, Oreg. 97223, are two of several incubator sources.
- g. Saylor Beal, St. Johns, Mich. 48879.
- h. Zeiss (W. H. Kessel and Company), 510 N. Dearborn Street, Chicago, Ill. 60410; Uni-tron Instrument Company, 66 Needham Street, Newton, Highland, Mass. 02161.
- i. Grand Island Biological Company, P. O. Box 68, Grand Island, N. Y. 14072; Microbiological Associates, 4813 Bethesda Avenue, Bethesda, Md. 20014; and Hyland Laboratories, 4501 Colorado Boulevard, Los Angeles, Calif. 90039, are three companies utilized by the Regional Poultry Research Laboratory, USDA, East Lansing, Mich.
- j. DIFCO, 920 Henry Street, Detroit, Mich. 48201.
- k. Colorado Serum Company, 4950 York Street, Denver, Colo. 80216.
- l. Barnstead Company, 225 Rivermoor Street, Boston, Mass. 02132. Mixed bed cartridge, #0808.
- m. Nutritional Biochemicals Corporation, 21010 Miles Avenue, Cleveland, Ohio 44128.
- n. Johns-Manville Company, 22 E. 40th Street, New York, N. Y. 10016.
- o. Consolidated Laboratories, Incorporated, P. O. Box 234, Chicago Heights, Ill. 60412.
- p. Technical Instrument Company, 348 Sixth Street, San Francisco, Calif. 94103.
- q. Flow Laboratories, 1206 Twinbrook Parkway, Rockville, Md. 20852.
- r. Cooke Engineering Company, Medical Products Division, 735 North Street, Alexandria, Va. 22314.
- s. ROBOZ Surgical Instrument Company, 810 18th Street N.W., Washington, D. C. 20006.

Use of the Hemacytometer

A Spencer bright-line hemacytometer is used. It is ruled into two areas of 9 mm.² each. These in turn are divided into nine squares, each with an area of 1 mm.² and each bounded by a group of three lines. The central square millimeter is the one used for counting cells. It is ruled into 25 groups of 16 small squares, each group separated by triple lines—the middle one is the boundary. The ruled surface is 0.1 mm. below the cover glass; hence the volume of fluid over 1 mm.² is 0.1 cubic mm. The number of cells in this 0.1 cubic mm. volume is then counted and the value multiplied by 10,000 to convert to cells per milliliter.

The procedure for counting is as follows: A clean cover glass is placed onto the supporting ribs of the counting chamber of a clean hemacytometer. The cell suspension to be counted is dispersed and a small amount is removed with a sterile capillary tube. The capillary is placed on the counting chamber at the edge of the cover glass. With this method, the fluid is drawn rapidly into the chamber. Excess fluid should be avoided. Either all 25 squares in the middle 1 mm.² are counted and multiplied by 10,000 or the four corners and one middle square are counted and multiplied by 50,000 (only 1/5 of the area counted). Cells on two sides of the outer boundary are included in the total count. Both sides of the hemacytometer are counted and an average is determined. The slide is cleaned, and the volume of cell suspension to add to the petri dishes to give the desired cell number is calculated. Fresh medium is added as needed to give the final volume of fluid in the petri dish.