RAPID, REVERSIBLE, ROUNDING AND AGGREGATION OF ROUS SARCOMA VIRUS-INFECTED, BUT NOT OF NORMAL, CHICKEN FIBROBLASTS INDUCED BY A PLASMA MACROMOLECULE

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Presented to the Faculty of Graduate Studies,
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of the Requirements for the Degree of
Master of Sciences

by

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Preface

I would like to thank my supervisor, Dr. Sam Balk for the use of his laboratory and the opportunity to do research.

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ABSTRACT

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Rous sarcoma virus (RSV)-infected chicken fibroblasts undergo a rapid (5 minute) rounding and aggregation after change to fresh plasma or serum containing culture medium. I have called this phenomenon the "medium change response" (MCR). No MCR, whatsoever, is observed in control cultures of normal chicken fibroblasts.

In low density cultures the MCR is essentially reversible; virtually all RSV-infected fibroblasts emerge from aggregates, re-attach and spread within 1-2 hours. In moderate or high density cultures, however, some infected cells no longer emerge from the aggregates that form after medium change. This failure of reversal of aggregation is responsible for the ultimate presence of large cell clumps in high-density cultures of RSV-infected fibroblasts that have been subjected to daily medium changes over a 4-5 day period.

The MCR occurs with media containing chicken plasma or serum, human adult or cord serum, calf serum or fetal bovine serum.

Ultrafiltration and dialysis studies indicate that the MCR is induced by a plasma macromolecule in the 50,000-100,000 molecular weight range. The MCR is not induced by the addition of chicken or human plasminogen.

The MCR ceases to occur after RSV-infected fibroblasts have been cultivated for 3 days in medium that contains 10^{-5}M leupeptin or 2 units/ml heparin. High density cultures of RSV-infected fibroblasts, prepared with these concentrations of leupeptin or heparin, manifest a flat, rather than a clumped, appearance. Neither leupeptin, at concentrations up to 10^{-3}M, nor heparin, at concentrations up to 100 units/ml, are capable of abolishing the MCR when added to cultures for the first time with fresh serum or plasma-containing medium. RSV-infected fibroblasts gave a partial MCR when cultivated for 3 days in medium that contained Epsilon-Amino Caproic Acid (EACA), Gamma-Amino Butyric Acid (GABA), soybean trypsin inhibitor, antipain, lysine, or Trasylol.

Cultured RSV-infected chicken fibroblasts appear to be distinguished from normal control fibroblasts, therefore, by the manifestation of a rapid, reversible rounding and aggregation in response to a plasma macromolecule. This response appears to involve a protease but does not appear to involve activation of plasminogen. Possible bases for the MCR are discussed as well as the potential significance of the phenomenon.

ABBREVIATION LIST

5' -AMP: Adenosine 5' Monophosphoric Acid

BHK: Baby Hamster Kidney Cell Line 21

3T3 Cells: Mouse Cell Line (Fibroblast Type Cells)

COFAL-Negative: Complement-Fixing Avian Leucosis Virus Antigen-Negative

Dbc3',5'-cyclic Amp: N^2, O^2- Dibutyrl Adenosine 3':5' Cyclic Monophosphoric Acid

EGTA: Ethylene Glycol Bis (B-Aminoethyl Ether) - $N_1N_1N^1{}_1N^1$- Tetraacetic Acid

EACA: Epsilon Amino Caproic Acid

GABA: Gamma Amino Butyric Acid

GIBCO: Grand Island Biological Company (New York)

"gs" Antigens: Group Specific

LETS: Large External Transformation Sensitive Glycoprotein

MCR: Medium Change Response

PEG-CF: Physiological Electrolytes and Glucose - Calcium Free

PMA: Phorbol-12-Myristate-13-Acetate

$pp^{60^{src}}$: Phosphoprotein 60,000 daltons produced by the src gene

RAV: Rous Associated Virus

RSV: Rous Sarcoma Virus

SEM: Standard Error of Mean

SC6: Synthetic Medium #6

SPF: Specific Pathogen Free

SV-40: Simian Virus - Strain 40 (DNA Virus)

TLCK: N-α-Tosyl-L-Lysyl Cholormethyl Ketone

TSSA: Tumor Specific Surface Antigen

Y-1: Mouse Adrenal Cortical - Tumor Cell Line

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INTRODUCTION

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Scope

Rous sarcoma virus infected chicken fibroblasts appear to be distinguished from normal control fibroblasts by the manifestation of a rapid, reversible rounding and aggregation in response to a plasma macromolecule. This response appears to involve a protease but does not appear to involve activation of plasminogen. Possible bases for this phenomenon are discussed as well as possible relationships to established properties of Rous sarcoma virus infected fibroblasts. The experiments in the thesis were designed to characterize this phenomenon occurring in Rous sarcoma virus infected chicken fibroblasts *in vitro*.

Literature Review

> "One of the great advances of 20th century cancer research was the experimental production of cancer with the use of specific physical, chemical and biological agents."
>
> Armin Braun (1)

In 1910 Peyton Rous, an American pathologist, had demonstrated that a malignant tumor of the chicken called a sarcoma (solid tumor of the connective tissues) could be transmitted from a diseased chicken to a healthy one by means of cell-free filtrates. This demonstration implied that a virus was responsible for the sarcoma (2). Alexis Carrel, demonstrated that primary explants of both normal tissue and malignant tumors of avian and mammalian origin could be cultivated in vitro in a homologous plasma clot with actual multiplication and growth of cells (3). He observed that during cultivation of certain human tumors (sarcomas and carcinomas) in clotted plasma, liquefication of the plasma clot occurred (3). Further investigation was carried out by Albert Fischer who found that primary explants of normal epithelial cells produce some liquefaction of chicken plasma clots and that there is an enhanced level of plasmin in cultured malignant cells such as Rous chicken sarcoma and Flexner-Jobling rat carcinoma (4). In the late forties a cellular agent had been discovered by T. Astrup that activated a fibrinolytic enzyme precursor called plasminogen (5). E. Reich and co-workers carried on Fischer's investigation on tumor-associated fibrinolysis using a cell culture system (6, 7, 8). They found that transformation of cultured fibroblasts of avian and mam-

malian origin by oncogenic viruses is associated with the production and release of a protease(s) that can convert plasminogen into plasmin. Plasmin, a fibrinolytic enzyme in serum, is capable of lysing a plasma clot.

Studies of Normal and Tumor Virus Infected Cells

One of the main objectives of experimental cancer research in the past and present is to understand the basic cellular mechanism(s) responsible for the neoplastic state. The use of tumor-inducing viruses has provided us a tool for studying this objective.

Citing Armin Braun (9):

> "A cellular mechanism that is now well established and that has been shown to account for the origin of certain tumor cells is one that involves the addition of new genetic information resulting from infection by the tumor inducing viruses."

These tumor-inducing viruses are broadly classified as oncogenic RNA viruses and oncogenic DNA viruses that are capable of inducing the neoplastic state in specific cells (10). The addition of appropriate new genetic information from an oncogenic virus to a normal cell may result in the conversion of that cell to a neoplastic cell, bringing about a heritable cellular change (11).

"Transformation" of Cells In Vitro

Normal control animal cells *in vitro*, following exposure to a specific tumor-inducing virus, can be converted into the neoplastic state (12, 13). When normal animal cells are exposed to a specific tumor-inducing virus they can undergo a variety

of characteristic changes. These cell culture changes, referred to collectively as "transformation", will be described later (12, 13, 14, 15, 16, 17). Which of these characteristics of the "transformed" state is required for maintenance and development of the neoplastic state is not clear. There is an ambiguous relationship between the existence of the neoplastic or malignant state and the established criteria of the "transformed" state. An example is SV-40 virus "transformed" human cell lines having a number of "transformation criteria", but which fail to produce tumors in athymic nude mice (18). Other examples are provided by strains of avian sarcoma viruses that do not "transform" cells in culture by any criteria, but produce sarcomas when inoculated in chickens (19). The above can be summed up by the following quotation of Samuel Balk (20):

> "...there are transformed cells that are not neoplastic and neoplastic cells that are not transformed."

Normal control fibroblasts, freshly isolated or from cell lines, that after being infected with a specific tumor virus, undergo metabolic, growth or morphological changes and remain viable can be called "transformed" cells (12). For example, RNA tumor viruses such as some avian and murine sarcoma viruses alter cellular characteristics and virus replication does not involve death of the infected cell. DNA viruses, such as polyoma and SV-40 alter cell characteristics but kill the cells for viral replication. However, "transformation" of cells infected with these DNA viruses can occur upon incomplete infections i.e. with viruses defective in the genes causing cell death, or in cells which are known to be non-permissive restrict-

ing the function of these viral genes (12). Often used in cell culture studies are the BHK21 cells line and 3T3 cell line which are non-permissive for polyoma virus and SV-40 virus respectively (12).

Most avian RNA sarcoma viruses are capable of converting normal connective tissues *in vivo* into neoplastic lesions within 48-72 hours of inoculation of a chicken (21). These same avian RNA sarcoma viruses are capable of converting normal fibroblasts *in vitro* to the neoplastic state or the so-called "transformed" state (14, 20, 21, 22). These virus-infected fibroblast-type cells *in vivo* are indistinguishable in morphology and other cellular characteristics from virus "transformed" fibroblast cells *in vitro* (21).

Criteria of "Transformation" of Cells In Vitro

Criteria associated with transformation will now be described. One major criterion is change in phenotypic expression of morphology of individual cells or of cell populations that accompany non-lytic infection by an oncogenic virus (12, 15, 17). Individual fibroblasts when "transformed" generally will have an altered morphology; an example is avian sarcoma virus infected chicken fibroblasts. When "transformed", these will convert from fan-shaped cells to a round or spindle shape (14, 23, 24). Other observable fibroblast structural changes include increased number of nuclei, nucleoli, increased nuclear to cytoplasmic ratio and chromosome aberration (15, 20, 21). As a population the cells form an irregular, rather than a regular cell pattern. The cells lose their parallel orientation

when "transformed" and have a tendency to heap up on one another, forming "criss-cross" patterns (25). Different oncogenic virus strains cause varying morphological changes in the cells (25).

Another phenotypic change is the alteration of cell surface architecture. For example, accumulation of hyaluronic acid and sulfated mucopolysacchrides in RSV "transformed" cells (26), increase of glycosyltransferase following RSV-infection of chick embryo fibroblasts (27), and alteration of glycolipid synthesis. An example of the latter is a decrease in membrane glycolipids with non-reducing terminals coupled with an increase of precursor glycolipids. This effect has been observed in both RSV and DNA virus "transformed" cells (28, 29, 30). Another alteration of transformed cells, particularly RSV-infected fibroblasts, that is associated with surface architecture is the presence of the extracellular matrix glycoprotein called fibroblast surface protein or fibronectin (LETS; Large External Transformation Sensitive glycoprotein). K. Yamada and co-workers have reported a decrease in cell surface fibronectin on cells "transformed" by oncogenic viruses or carcinogens and on cultured tumor cells with exceptions in each case (31). For example, RSV-infected chick embryo fibroblasts have a considerably less amount of surface fibronectin than normal counterparts (31, 32). Decreased surface content of fibronectin along with decreased cell to cell, and cell to substrate adhesiveness, changes in cell morphology and cell surface architecture in tumor and "transformed" cells may be viewed as pleiotropic characteristics

of the process of metastasis (31, 33).

Chick embryo fibroblasts infected with a mutant Rous sarcoma virus that is temperature conditional for "transformation", were significantly less adherent than their normal counterparts following a shift to a permissive temperature (34). This observation of M. Weber demonstrates that altered adherence is specifically associated with "transformation" rather than simply with the virus infection (34, 35). From various studies, correlations of decreased adherence of transformed cells with changes in internal cytoskeletal structures involving microfilaments and microtubules have been made (34, 36, 37). There is evidence of a reduction in the total amount of actin microfilaments and microtubules in virally "transformed" cells (38, 39). Microfilament bundles and microtubules were found to disassemble and assemble reversibly at restrictive and permissive temperatures, respectively, when cells were infected with temperature-sensitive mutant Rous sarcoma virus (34, 40). This information suggests that these cytoskeletal structures are involved in both structural and functional changes that occur in "transformed" cells. Intricate networks of cytoskeleton structures (microfilaments and microtubules) in fibroblasts are known to be lying just under the cell membrane and are responsible for cell shape and movement (41, 42).

Other changes of "transformed" cell membrane surfaces are: increased plant lectin agglutinability, a greater mobility of lectin binding sites (indicating a more fluid membrane) and new surface antigens. This latter characteristic is either

specified by a virus or a modified cell antigen (43, 44, 45, 46, 47). Plant lectin agglutinability is not a specific property of "transformed" cells. When normal counterparts are trypsinized they have an increased agglutinability similar to "transformed" cells. This indicates that the normal cell lectin-surface-receptor-sites are being masked while the transformed sites are bared (48). A unique antigen called the "tumor-specific cell surface antigen" (TSSA) has been detected in cells "transformed" by any of the Rous sarcoma virus strains (49).

Oncogenic viral "transformation" of fibroblasts causes alteration of growth behaviours; reduced sensitivity to density dependent inhibition being one of them (12, 15, 17, 20). Density dependent inhibition is a cell culture phenomenon in which proliferation of normal cells is restricted by high culture density and/or culture medium depletion (50). Those fibroblasts that show density dependent inhibition in confluent cultures have reached a saturation density as the cells enter a G_0 phase. The saturation density varies as to the fibroblast line, strain and as to whether the cells are or are not freshly isolated (12). Some populations of "transformed" cells acquire the ability to form colonies when suspended in medium containing agar or methocel (12, 14, 15, 51, 52). H. Rubin and J. Bader have reported that RSV-infected chicken embryo fibroblasts, but not normal fibroblasts can form colonies when suspended in agar (51, 52).

Other altered growth behaviours of "transformed" cells include a reduced serum requirement for growth and unlimited

number of divisions in culture (53, 54, 55). S. Balk et al observed that RSV-infected chicken fibroblasts have the capacity to proliferate in a culture medium of reduced divalent cation concentration (calcium and magnesium) which may be related to the ability of neoplastic cells to proliferate autonomously (56).

Metabolic changes have been associated with transformation; these include increase in certain amino acids, glucose and other sugars uptake (57). Increase in glucose uptake has been associated with increased aerobic glycolysis and reduced respiration in "transformed" cells and tumor cells (58). It has been suggested that neoplastic cells may proliferate due to a deficiency in the cellular respiratory function (59). Increased glycolytic pathway enzymes and lactic acid synthesis have been found in Rous sarcoma virus "transformed" cells under certain conditions (57).

Fibroblasts of avian or mammalian origin "transformed" by RNA or DNA tumor viruses have been associated with development of increased fibrinolytic activity (6, 7, 8). J. Unkeless et al found that a protease released by virally "transformed" cells in culture converted serum plasminogen to plasmin, a serum fibrinolytic enzyme (6). Plasminogen activator(s) levels are high in avian and mammalian cells "transformed" by oncogenic and chemical carcinogens, in specialized normal and tumor cell lines, neoplastic tissues, macrophages and developing tissues in vivo (6, 7, 8, 60-67). It is not clear why there is a high level of plasminogen activator production in "transformed" cells in vitro and in some malignant tissue in vivo. B. Wolf and

A. Goldberg show low levels of plasminogen activator in some cloned "transformed" cells and have suggested that plasminogen activator is not essential for expression of "transformation" (68). Plasminogen activator may activate other proenzymes besides plasminogen, that could be important in the "transformed" or neoplastic state. There are other serine proteases that have been reported to be released by malignant mammalian cell lines and virally "transformed" avian fibroblasts (69, 70).

Another property of virally "transformed" cells is cellular tumorgenicity when the cells are injected into congenitally athymic nude mice (16, 17). This property has been used as an indicating system to determine malignant "transformation" of cells cultured in vitro (17). The use of athymic nude mice as indicators of tumorgenicity of cells is debatable. In virally "transformed" cells of established cell lines, one does not know if the tumorgenicity of cells is due to the virus or selection resulting from the culture (16).

Avian Leukemia-Sarcoma Complex of Viruses

To obtain the neoplastic state for comparison to normal counterparts, fibroblasts cultured from pectoral muscles of eight-week-old cockerels were infected with the Schmidt-Ruppin strain of the Rous sarcoma virus, a member of the avian leukosis-sarcoma complex of viruses (25, 103). These viruses: a) Have group specific antigens, which are called "gs" antigens, found in the core of the virion, demonstrated by complement fixation (25); b) have RNA-dependent DNA polymerase; c) have a natural host range restricted to birds, although under experimental

conditions may infect mammals or mammalian cells (10); d) cause a productive infection with no direct cell killing in genetically sensitive avian cells although some strains may cause a non-productive infection of mammalian cells e) may be strong, weak, and "non-transforming" just as there are strong, weak and non-oncogenic viruses (103, 104). f) Are divided into subgroups A, B, C, D, E and F based on envelope characteristics, autogenous interference to infection, and neutralization by antibodies (103). Another classification system is by strains and presence of genes for "transformation" (104). An example of an avian RNA virus without a "transforming" gene is the Rous associated virus (RAV), which does not transform infected cells (102). An avian RNA virus that has a "transforming" gene is the Rous sarcoma virus (104).

The leukomagenic members of the complex group are involved in causation of leukemias (i.e. myeloblastosis) and lymphomas (i.e. lymphoid leucosis). The sarcomagenic members of the complex group are involved in the causation of sarcomata (i.e. fibrosarcoma) (14). Cells infected by avian sarcoma viruses that become neoplastic and/or "transformed", are the results of the expression of the viral sarcoma or "src" gene (25, 104, 105). In this viral gene, protein product expression has been characterized as a phosphoprotein, with a protein kinase activity and has been designated $pp60^{src}$ (106, 107). There may be a possible relationship of protein phosphorlyation by the src gene product and the "transformed" and/or neoplastic state of the avian RNA virus infected host cell. Characteristics such as protease production after "transformation" may be due also to src gene

expressions. Coincidentally, normal uninfected avian cells contain a highly conserved nucleotide sequence called the sarc gene in their DNA and RNA that is related to the viral src gene (108). Further work is needed to find the function of both these genes and their relationships.

Rous Sarcoma Virus Chicken Fibroblast System

The phenomenon of rapid, reversible, rounding and aggregation of RSV-infected chicken fibroblasts in response to the addition of serum or plasma containing culture medium were observed by S. Balk during his experiments involving control of proliferation of chicken fibroblasts (14). In studying this phenomenon I have used the RSV-infected chicken fibroblast system established by S. Balk. This system was used because the efficient conversion of normal chicken fibroblasts to the neoplastic state ("transformed"), so that a valid comparison can be made of the virally converted and normal control cell populations (102). Fibroblasts were cultured from the pectoral muscles of young adult chickens (8 weeks old) rather than chick embryos because of a greater certainty as to the origin of these cells. In this study I have used freshly isolated cells, rather than established cell lines, to avoid artifacts due to abnormalities that have been found in latter.

Proteases in Biological Fluids

Proteolytic enzymes in biological fluids released from tissues play an important role in biological control mechanisms serving in many diverse functions (71). Proteases participate in complex biological phenomena such as in gamete formation,

fertilization, cell migration, tumor invasiveness, intra-and extra-cellular protein turnover, morphogenesis and metamorphosis (72). One type of proteolytic activity is called limited proteolysis, involving conversion of an inactive precursor of a protein (zymogen) to an active form (71). This is an essential physiological regulatory mechanism in biological fluids and is found in the enzyme cascades of blood coagulation, complement activiation, fibrinolysis and kinin generation (72). Zymogen activation in the case of plasminogen conversion to plasmin by plasminogen activator, or factors of the enzyme cascade system converting prothrombin to thrombin depend on threshhold events initiating and activating proteases (5, 71). This activation mechanism usually encompasses several characteristics; best observed are; amplification, rapid response, sensitivity to effector concentration and irreversibility (71). B. Hartley classified proteases in animals into five major sets based on mechanisms and inhibitors. These are, with their identifying active feature: metalloexopepidases containing Zn^{2+}, sulfhydryl proteases with CySH, acid protease operating at an acid pH optimum, metalloendopeptidases utilizing Zn^{2+} and Ca^{2+}, and serine protease with an active serine group (73). The majority of the limited proteolytic-type enzymes in biological control mechanisms are from the serine protease class. They share similar amino acid sequences, homologous tertiary structures and catalytic mechanism of action and are inhibited by serine protease inhibitors (72, 73). The active site of a serine protease contains a specific serine residue, participating in the formation of an intermediate

ester with an acyl group of a substrate (74). The class of serine proteases includes many components of the blood coagulation cascade system such as activated factors VII, IX, X, XI, XII and thrombin (75). There are serine proteases secreted by the pancreas involved in protein digestion such as trypsin and chymotrypsin (71).

Another serine protease is plasmin, a fibrinolytic enzyme with a trypsin-like specificity capable of hydrolyzing peptides and proteins at arginyl and lysyl peptide bonds, basic amino acid esters, and amides (72). Activation of plasminogen to plasmin in mammals can occur by several activators; these are urokinase from kidneys, streptokinase (Streptococcus bacteria exotoxin) and tissue activators (76). In avian plasma the only known plasminogen activator(s) is the tissue activator(s). Streptokinase or mammalian urokinase will not activate avian plasminogen (71). In mammals activated Hageman factor or fragments can activate plasminogen pro-activator which in turn activates plasminogen activator (77, 78). Hageman factor is one of the key initial enzymes which starts the Hageman factor-dependent pathway. This factor can initiate the blood coagulation cascade, fibrinolysis pathway and kinin generation (77). See Appendix A for a description of the Hageman factor pathway. In avian plasma, the intrinsic coagulation system is deficient by mammalian standards but the extrinsic system is active and efficient (79). Factors IX (thromboplastin) and XII (Hageman factor) have been reported absent or in very small amounts in avian blood (79, 80, 81). Avian blood coagulation appears to be dependent on the ex-

trinsic clotting system involving the release of tissue thromboplastin (79, 82).

Plasminogen Activator Role in Cells

Increased levels of plasminogen activator were discussed before in reference to "transformation". Plasminogen activator(s) is also secreted by normal cells in lower amounts such as in mammalian heart, lung, kidney, capillary endothelial cells and activated macrophages (72). Plasminogen activator is also found in differentiating and developing cells. Increased levels have been found in granulosa cells *in vivo* near the time of ovulation from follicles destined to ovulate in response to gonadotropins (83, 84). Increased plasminogen activator levels have been found in trophoblasts and parietal endoderm cells during early embryogenesis. This increase coincides with the invasive phase of trophoblasts and the differentiation of parietal endoderm cells during implantation and growth (63). Plasminogen activator levels were found to increase during mammary gland involutions in response to hormones. The basis for the increase has been related to tissue remodelling (62). Increased plasminogen activator production during proliferation and development of neoplastic, hyperplastic, and euplastic tissue, has led to suggestions that plasminogen activator functions in altering the interaction between cells involving destruction of the cell matrix, cell remodelling and cell migration (62, 63, 83, 84). Plasminogen activator(s) and several other serine enzymes have been found to be released from cultured human and avian cells of neoplastic origin (4, 64).

Many studies have been done trying to show that the release of plasminogen activator is a biochemical criterion for malignancy. Now it is known that plasminogen activator(s) is not specific for malignant cells (64). A study was done on primary human neoplasms compared to normal tissues, *in vitro* to determine the differences in plasminogen activator levels. The results showed that there is an increase in plasminogen activator in human neoplasms of mesenchymal and neural origins compared to corresponding normal cells. But from human neoplasms of glandular or lining epithelium compared to corresponding normal cells no consistent correlation could be made (64). Very little is known on the initiator(s) of plasminogen activator(s) release in tissue. A study by S. Strickland has shown that gonadotropins induce increased levels of plasminogen activator(s) in ovarian granulosa cells (84). Very little is known about the characteristics and function of the other serine enzymes secreted by "transformed" cells *in vitro* and malignant cells *in vivo*.

Chicken embryonal fibroblasts "transformed" by Rous sarcoma virus have been reported to release another protease, beside plasminogen activator, which has a collagenolytic activity (69). This protease is released by "transformed" cells at confluency and subconfluency while only in normals at subconfluency. This enzyme does not appear to be a virally "transformed" cell specific enzyme.

Protease Inhibitors

In correlating the association of some tumors *in vivo* and "transformed" cells *in vitro* with increased serine protease

secretion, serine protease inhibitors have been used as a tool to determine the functions of these secretions. There are inhibitors of physiological activators of proteases that are present in the blood circulation such as α-2-macroglobulin, a protease inhibitor in human serum (72, 85). Protease inhibitors that are specific to serine class enzymes are usually anti-fibrinolytic agents, and have been used clinically (86). Serine protease inhibitors used in tissue culture can be classified into several categories: a) Those that are substrate anologs and inhibit by competing with the natural substrates of the proteases and esterases; an example is N-α-Tosyl-L-Arginyl Methyl Ester (TAME) (87); b) Those that are substrate analogs that react covalently with trypsin-specific enzymes such as N-α-Tosyl-L-Lysyl Chloromethyl Ketone (TLCK) (88); c) Those that form poorly dissociating complexes with the protease, for example soybean trypsin inhibitor, lima bean trypsin inhibitor and benzamidine (67, 72, 86). The inhibitors described in "a & b" do not produce good reproducible results in tissue culture due to their instability, reactions with other serum components, and the sensitivity of cells to them (88, 89). Trasylol (generic name) is a bovine pancreatic enzyme inhibitor capable of inhibiting enzymes in the kallikrein-kinin and fibrinolysis system (86, 90).

There are serine protease inhibitors that are used clinically as anti-fibrinolytic agents for example Epsilon-Amino-Caproic Acid (EACA) (86), a lysine anologue which functions as an anti-fibrinolytic agent by inhibiting plasmin and plasminogen activation (91). Heparin is a sulphated mucopolysacchride that is used clinically as an anticoagulant (93, 94). Heparin

along with a co-factor, antithrombin III (alpha-2-globulin) is an anticoagulant which functions by inhibiting thrombin from converting fibrinogen to fibrin. Heparin is also capable of inactivating activated factors XII, XI, X and IX (93, 95).

Cellular proteases have been implicated to be involved in growth control and development (96). Studies using protease inhibitors on "transformed" cell lines have shown variable results from cell line to cell line (88, 89, 97, 98). One can argue that these studies involving comparison of a "transformed" cell line to a normal cell line such as 3T3 cell line to SV-40 3T3 cell line are not rigorous. The "normal" 3T3 cells are a cloned cell line while the SV-40 virus infected 3T3 cells are another clonal cell line consisting of only a selected population of 3T3 cells that can be infected by SV-40 virus as in the case of most DNA tumor virus cell line systems (14). Such cell lines cannot be compared rigorously because of possible characteristics which reflect differences between the bulk of cells in the "normal" population to one selected group of cells in the virus-infected cells. In an RSV-infected primary chicken fibroblast system the virus can infect all the fibroblasts, thus a rigorous comparison can be made between virus infected and normal homogenous cell population.

Protease inhibitors have also been known to revert some characteristics expressed in "transformed" cells back to their normal characteristics (99, 100, 101). M. Weber found that RSV-infected chick embryo fibroblasts, when treated with a protease inhibitor such as TLCK, were found to increase in adhesiveness to the culture surface and assume a flat morpho-

logy just as their normal counterparts (100). He suggests involvement of a trypsin-like enzyme, possibly plasmin, in the decreased adhesiveness and rounded morphology of virally "transformed" cells (99). Studies were done growing RSV-infected chick embryo fibroblasts in plasminogen-free serum. These cells showed the flattened morphology similar to normal cells after a period of 5 days (100). Weber suggests another protease may be involved in the "transformed" phenotype (99, 100).

MATERIALS AND METHODS

MATERIALS AND METHODS

Incubation Conditions

The cultures were incubated in hydrated isolettes in a 41.9°C room. The hydrated isolettes were controlled atmosphere culture chambers (Bellco Glass Inc., Vineland, New Jersey) that were made of plexiglass, gas tight, with perforated removable shelves and controlled gas inlet and outlet flow valves. These chambers were placed on Bellco rocker platforms and could be kept static or on a rocking speed. The chambers were constantly perfused with hydrated 95% air, 5% CO_2 atmosphere. The 5% CO_2 component of the gas mixture was an important determinant of the culture medium pH, which was 7.4. The CO_2 in the culture chamber is monitored with a Fyrite CO_2 indicator (Bacharach Instrument Company, Pittsburgh, Pa.).

The gas mixture was hydrated to prevent evaporation of culture medium from the dishes. The humidity was kept at about 100%, this was obtained by passage of the gas mixture through two serially connected carboys of water (Fisher Co., Canada).

Labware

The glass labware and Nalgene labware were sterilized in an autoclave prior to use. The centrifuge tubes, pipettes and culture dishes were sterile and disposed immediately after usage. Lux Contur tissue culture dishes (Lux Scientific Corporation, Newbury Park, Calif.) were used for experiments and to grow cells. These dishes were made of polystyrene, ultraviolet radiation sterilized, had good optical clarity, designed

for optimal cell attachment, and had a flat distortion-free cell growing surface. The dishes were designed to prevent aggregation of cells at the edge of the dish. For growing stock cultures 60 x 15 mm dishes were used with 4 ml, of culture medium added to each dish. In experiments 35 x 10 mm dishes were used with 2 ml, of culture medium. Falcon dishes (Oxnard, Ca.) were used in trial experiments, to compare against Lux Contur dishes.

Preparing, Passaging, and Infecting of Cell Cultures

The following procedures were developed by S. Balk et al (14, 59).

Preparation of Primary Cultures

Pectoral muscles were excised under sterile conditions from a 8 to 9 week-old, male, Specific Pathogen-Free (SPF) COFAL-negative white leghorn chicken. (Spafas, Inc., Norwich, Conn.). Betadyne (water soluble iodine antiseptic) and 70% ethyl alcohol were used to sponge the breast area, for sterilization. Briefly, the excision process is as follows: Feathers above the pectoral muscle were removed, the outer skin was cut distally to the pectoral muscle and pulled dorsally with 3 sterile hemostats, and a rectangular piece of pectoral muscle (5 cm x 5 cm x 1 cm) weighing approximately 20 grams was excised from both sides using a sterile scalpel with a No. 22 blade. These pieces of pectoral muscles were then immediately placed in a sterile beaker containing 50 ml of calcium free physiological electrolytes with glucose medium (PEG-CF), (Appendix B) in an ice bath. Under sterile conditions the pectoral muscles were

then shredded with 4 No. 11 scalpel blades set on a common handle, and put into a 125 ml trypsinization flask (Bellco Inc., Vineland, N.J.) with a magnetic stir bar. The shredded muscles are then trypsinized with a trypsin medium (Appendix B) consisting of 0.10% trypsin (Grand Island Biological Company, Grand Island, New York) in a synthetic medium containing electrolytes (PEG-CF). Using this trypsin medium six 10-minute trypsinization cycles were done with different amounts of medium for the cycles (in order), 70, 70, 60, 60, 45 and 45 ml. After each cycle the medium was discarded due to high viscosity from actomyosin and fragments of myotubules. One final 10-minute cycle was then done, with 45 ml of trypsin medium. After this cycle the medium from the flask was poured through 2 sterile gauze sponges (2 x 2 - 8 ply) into a 50 ml disposable sterile polypropylene centrifuge tube (Kimble, Owens-Illinois, Pittston, PA.). The tube was then centrifuged @ 2500 x g, @ 1°C for 30 minutes with the brake set at maximum, using a Beckman, model J-6 centrifuge. The supernate was then discarded and the pellet was suspended in 10 ml medium SC6 with heat-inactivated plasma (see methods and material, culture medium) using 21 triturations with a 10 ml pipet, then a further 7 ml of medium SC6 with 5% heat-inactivated plasma was added to the suspension. The diluted suspension was agitated and used to seed 2-60 x 15 mm Lux Contur dishes (4 ml/dish).

The following day the cells were viable and firmly attached to the culture dish with debris floating in the culture medium. The old culture medium was removed and replaced with fresh medium with SC6 with 5% heat-inactivated plasma and changed every second day from then on. On the 4th-6th day the

primary cultures had reached confluency, fibroblasts being the predominant mononuclear elements. Also dispersed in the confluent fibroblasts were myotubules which had formed as a result of extensive fusion of myoblasts. The myotubules present are end stage structures and are lost when the culture is passaged.

Preparation of Secondary Cultures and Infection by Rous Sarcoma Virus

Passaging Primary Cultures to Secondary Cultures

On day 6, the confluent primary cells were passaged into secondary cultures. The passaging procedures were as follows: Old growth medium was removed by aspiration and replaced with 2 ml of 0.025% trypsin (GIBCO) in PEG-CF. The dishes were put in the warm room into an isolette set on a rocker platform (2 cycles per minute). After 30 minutes of trypsinization most of the cells rounded up and detached from the culture dish surface. These cells were collected using 9 gentle wash triturations to remove all cells, with a sterile plugged Pasteur pipet and dispensed into a 50 ml centrifuge tube (Kimble). They were centrifuged @ 300 x g, for 10 minutes at 25°C, with the brake set at 1. The medium was then discarded and the pellet was suspended with 10 ml medium SC6 with 5% heat-inactivated plasma with 21 triturations. The cell suspension was diluted to 3.0×10^4 cells/ml in medium SC6 with 5% heat-inactivated plasma seeding medium and 4 ml was added to each of 6-60 mm Lux dishes. For secondary cell cultures to be grown in chicken serum, medium SC6 with 5% Colorado chicken serum was substituted for medium SC6 with 5% heat-inactivated plasma in the seeding medium.

Infection of Secondary Cultures with Rous Sarcoma Virus

The cells were incubated for about 24 hours after passage. Then the growth medium was aspirated and replaced with 2.0 ml per dish of Schmidt-Ruppin Rous sarcoma virus inoculum to 3 secondary culture dishes and 2.0 ml of control inoculum was added to the remaining 3 dishes. The virus inoculum was culture fluid medium SC6 with 5% heat-inactivated plasma from densely infected tertiary cultures and the control inoculum was from respective tertiary normal cell cultures. The inoculums were stored in plastic 10 ml tubes (Falcon) at approximately -65°C and thawed out in a 42°C water bath upon immediate use. Similarily, inoculums were made for secondary cultures grown in medium SC6 with 5% Colorado chicken from infected and non-infected tertiary cultures grown in medium SC6 with 5% Colorado chicken serum. After the culture medium containing inoculum had been added the dishes were put back in the warm room for 3 hours, in an isolette rocking 1 cycle per minute. The inoculums were removed and 4 ml of fresh culture medium per dish was added and changed every 2nd day. The secondary cultures were grown for 5 days at which time the Rous sarcoma virus-infected and normal cells were confluent. The infection was complete in 2-3 days. Detection of complete infection can be visibly observed, when the infected cells have a spindle-shape morphology, smooth surface and refractile appearance. Similar morphologic changes have been observed in chick embryo and chicken fibroblasts infected with Schmidt-Ruppin Rous sarcoma virus (38, 35). The normal fibroblasts are fan-shaped with a ruffling membrane appearance.

Preparation of Tertiary Normal and Infected Cultures

Passage of secondary normal and Rous sarcoma virus-infected fibroblasts was by the same method as passaging of primary fibroblasts, except for procedures after suspension of the cells. The cells were suspended separately (normal and RSV-infected) in the same growth medium used for secondary cultures. After suspension the cells were diluted into seeding growth medium: The normal cells were diluted in the seeding growth medium to 6.0×10^4 cells/ml. The RSV-infected fibroblasts were diluted in the seeding growth medium to 3.0×10^4 cells/ml. The seeding growth medium was kept in Nalgene bottles and agitated using right angle shakes every so often during seeding in order to get an even distribution of cells. For experiments 35 mm Lux dishes were seeded by pipet, adding 2 ml of medium per dish. The dishes were immediately put into the warm room in the isolettes. Next day the tertiary cultures were changed to test medium and chaged on day 2 and every day until the termination of the experimental trial. If tertiary cells were needed for quarternary cell cultures, large 60 mm Lux dishes were seeded instead of smaller ones. If necessary, passaging of tertiary to quarternary cultures for experimental trials was exactly the same as for passaging secondary cultures. All of the experimental trials were as tertiary cultures.

Preparation of Sera and Plasma

The growth mediums for stock culture dishes and experimental trials were medium SC6 (Appendix C) with 5% Colorado chicken serum (Colorado, Denver) or fresh heat-inactivated chicken plasma.

Commercial Sera

For experimental trials, Colorado chicken serum was used mainly instead of GIBCO (New York) chicken serum, as the cells seemed to grow slightly better in the former. Commercial sera used for test trials were, human sera (GIBCO, non-reactive for HBs Ag by RIA), fetal bovine serum and calf serum (GIBCO). The sera were kept frozen at -20°C and thawed when needed. Human placental cord serum was a gift from Dr. M. Ray (Pediatrics, Winnipeg, Health Science Centre).

Blood Collection for Preparation of Chicken Plasma and Serum

In preparing plasma and serum from freshly collected blood, methods were used that were developed by S. Balk. Mature cockerels (1 year), specific pathogen-free, Complement-Fixing Avian Leucosis Virus Antigen-Negative (COFAL-negative) were bled from the wing veins. Alternate wing veins were bled on alternate weeks to avoid damage to the veins. The cockerels were not given food 17-20 hours before being bled, thus allowing a yield of clear plasma, relatively free of visible lipid. Using a Butterfly #18 infusion set, 19-6 diameter (Abbott Ireland Ltd., Sligo, Republic of Ireland), the needle was inserted into the vein. A period of 10-15 seconds were allowed for the vein to contract around the needle, then the adapter end was snipped off. Forty drops of blood are allowed to run through, and discarded to clear possible tissue thrombroplastins at the site of needle insertion. The blood was collected in sterile, siliconized unstoppered 100 x 16 mm, 4700 Vacutainer tubes (Becton, Dickinson & Co., Mississauga, Ontario), that were agitated constantly while being held

in an ice water bath. Six to twelve birds were bled at a time in this manner, about 2 tubes per bird with a time reference of about 60 seconds per tube. The blood was immediately pooled (after each bird is bled) in a sterile, Nalgene bottle placed in a covered ice bucket full of ice water.

The above methods are basic procedure used in collecting blood for preparing heat-inactivated plasma, heat-inactivated serum and serum from clarified plasma.

Preparation of Heat-Inactivated Chicken Serum

In preparing heat-inactivated serum, 50-60 mls of blood was clotted in a 2-litre sterile, roller glass bottle placed on rollers (Bellco Glass Inc., Vineland, N.J.) set for 4 cycles per minute in the warm room (41.9°C). The clotting process was complete in about 1-2 hours, then the clot was dislodged with a sterile pipet and left in the warm room for additional 3-4 hours (total of 5 hours in the warm room). The expression of serum was complete after 5 hours with little hemolysis taking place. Then the serum is centrifuged at 2,750 x g for 10 minutes and placed in a 56°C water bath for 35 minutes to be inactivated. The chicken serum inactivated is then frozen at -20°C until needed.

Preparation of Heat-Inactivated Chicken Plasma

In preparing heat-inactivated chicken plasma pooled blood was dispensed into sterile Nalgene conical centrifuge tubes (20 mls per tube) and centrifuged at 700 x g for 20 minutes at 4°C, brake set at 1. The plasma was then poured off into fresh

Nalgene tubes (40 mls per tube) and centrifuged 700 x g for 30 minutes. Again the plasma was poured off and centrifuged at 5,000 x g for 30 minutes. This last step is repeated, to give a total of 4 centrifugations. The procedure of centrifugation removed the formed elements from the blood. The clarified plasma was then pooled in a Nalgene bottle, dispensed into stoppered Vacutainer tubes and placed in a 56°C water bath for 35 minutes. This procedure formed a copious fibrinogen precipitate. The clarified plasma was then poured off in 50 ml centrifuge tubes, frozen and thawed three times and then spun at 2,750 x g for 10 minutes, 4°C, to remove any further precipitate. This plasma is then referred to as heat-inactivated plasma (heat-defibrinogenated plasma).

Preparation of Serum from Clarified Chicken Plasma

In preparing serum from clarified chicken plasma, the procedures for preparing plasma were used and after the fourth centrifugation the plasma was poured into a sterile (20 ml) polycarbonate centrifuge tube with a screw cap. The tube was placed in the warm room (41.9°C) for 18 hours, at the end of this period a thick clot had developed. The prolonged clotting period allows a good serum expression of the clotted plasma and exhaustion of the blood coagulation enzyme factors. The tube is then centrifuged in a Beckman model L5-65 (T:60 rotor head) ultracentrifuge at 110,000 x g for 2½ hours. This ultracentrifugation converts the thick plasma clot sheet into a small button at the bottom of the tube. The supernatant-serum from the clarified plasma is then frozen until needed.

Preparation of Heparinized Chicken Plasma

In preparing heparinized chicken plasma the process was exactly the same as for plasma except in the blood collection procedure. For heparinized plasma, 9.9 mls of blood was collected in 4700 Vacutainer tubes (marked at 10 ml level) containing 0.1 ml of 2000 units/ml heparin sodium salt (Organon, Canada Ltd., Toronto). The blood collected has a hematocrit value of 45-50% and a heparin concentration of 20 units/ml. When centrifuged for plasma preparation as described above, the final heparin concentration in the clarified plasma was approximately 40 units/ml. In culture medium used for experiments consisting of 95 parts synthetic medium 5 parts heparin 40 units/ml plasma, the final heparin concentration in the culture medium was 2 units/ml.

Preparation of Chicken Serum Ultrafiltrate

The method for commercial chicken serum ultrafiltration was done as described by AMICON, Mass. (Product Bulletin 210, May 1968). A number 8 dialysis tubing (Fischer Scientific, Pittsburgh, Penn.) was used. This tubing has a width of 1 centimeter when flat and dry, and has the ability to retain material of molecular weight 12,000 or higher. The tube was boiled in glass distilled water for thirty minutes. One end of the dialysis tubing was firmly knotted and suspended in a 2-litre vacuum flask by means of a glass funnel in a one-holed stopper. The funnel was filled with GIBCO chicken serum to allow a continuous drainage into the knotted dialysis tubing while a vacuum (1551 torr) was applied to the flask. The ultrafiltration was carried out in a cold room (4°C). After 24 hours, the serum ultrafiltrate

was collected from the vacuum flask and sterilized with 0.45u millipore filter in a Swinnex adapter.

AMICON Serum Filtration

Human and chicken serum (GIBCO) was fractionated to determine an approximate molecular weight range of the medium change response inducer. A Model 10 type AMICON ultrafiltration pressure cell (AMICON Corporation, Lexington, Mass.) which has a magnetic stir bar, 10 ml maximum process volume and filter selection diameter of 25 mm was used.

For filtration fractions of molecules under 50,000 dalton a 25 mm Diaflo (AMICON) XM-50 filter capable of retaining macrosolutes larger than 50,000 dalton was used. Prior to use the filters were washed and soaked overnight ($4^{o}C$) in double distilled H_2O to remove glycerine plasticizers. The filter was placed in the AMICON cell with the shiny side towards the serum to be filtered. Seven ml of serum was added to the cell and was replaced to this level during the fractionation, continually until total of 28 mls of serum had been poured in the cell chamber. A gas pressure of 2.8 kg/sq cm was applied with N_2 gas. The filtration was carried out in a cold room ($4^{o}C$). Total time of filtration was 3 hours (approximately 10 ml per hour). Both serum filtration effluent and retentate were used immediately for experiments.

In fractionating serum for molecules less than 100,000 dalton the XM-50 filter was replaced with a XM-100 filter. The Diaflo XM-100 filter retains molecules larger than 100,000 dalton. Exactly same procedures were followed as for the XM-50 filtra-

tion, except that the pressure was reduced to 0.7 Kg/sq cm N_2 gas. Both serum filtration effluent and retentate were kept and used immediately. For more detail instruction of AMICON filtration refer to the Product Bulletin 210, May 1968, AMICON Corporation.

Preparation of Dialyzed Chicken Serum

GIBCO chicken serum was dialyzed using a number 8 (Fischer) dialysis tubing, as was used for the ultrafiltration. The tube was boiled in glass distilled H_2O for thirty minutes. One end of the tube was firmly knotted, then 2 ml of serum was poured in, and approximately 1 centimeter above the serum miniscus another knot was tied. This small cylinder was then suspended in a beaker containing 2000 ml of 0.9% saline solution. The serum was dialyzed in the cold room ($4^{o}C$), with a magnetic stir bar used to stir the saline solution. After every 24 hours for 3 times the saline solution was removed and replaced with 2000 ml fresh solution. After the third time period the dialysis tube was removed from the saline solution and the dialyzed serum was drawn up by a syringe. The dialyzed serum was then sterilized using a 0.45 u millipore filter.

Plasminogen-free Chicken Serum Preparation and Plasminogen Isolation

Chicken plasminogen was prepared by affinity chromatography as was plasminogen-free chicken serum, by the methodology of D. Loskutoff (109). The same lot of commercial chicken serum was used in the preparations. Deplasminogenated chicken serum was 99% plasminogen-free, as judged by direct fibrin plate assay using the plasminogen activator present in culture medium con-

ditioned by RSV-infected fibroblasts. Plasminogen was isolated from 50 mls of chicken serum and kept in phosphate buffered saline.

Five units of human plasminogen was obtained from SIGMA, Missouri. A unit description defined by SIGMA is: 1 unit will produce a Delta A_{275} of 1.0 in 20 minutes at pH 7.5 at 37°C, when measuring perchloric acid soluble products from α-casein in a final volume of 5.0 mls.

Preparation of $Al(OH)_3$ Absorbed Heat-Inactivated Chicken Plasma

The addition of $Al(OH)_3$ (Amphojel gel, Wyeth Ltd., Toronto) to heat-inactivated plasma will remove most of prothrombin, factor X, factor IX and factor VII present in the plasma (110). A 1:100 dilution was made in a 50 ml centrifuge tube (Amphojel get to heat-inactivated chicken plasma). The tube was put on a Bellco rocker platform set at 16 cycles per minute 2 x 30 minutes. After the first 30 minute shaking period the tube was spun at 5000 x g for 10 minutes and then put back on the rocker platform for 30 minutes. The absorbed heat-inactivated chicken plasma was used without further preparation.

Culture Medium

Culture medium was made up of 95 parts of synthetic medium 6 (SC6, Appendix C) with 5 parts chicken serum or heat-inactivated chicken plasma for growing stock and experiment trials. Calcium content of heat-inactivated chicken plasma or chicken serum is 2.5 mM. In culture medium SC6, calcium free with 5% chicken serum or heat-inactivated plasma the calcium

concentration would be 5% of 2.5 mM (0.125mM). The calcium concentration of SC6 is 1.2 mM, this is made by adding 100 mM $CaCl_2$ concentrate to SC6, calcium free (Appendix C). Very low calcium concentration medium ($\sim 10^{-5}$M) was prepared by combining 90 parts of calcium free synthetic medium, 5 parts of 2.5 mm ethylene glycol bis (B-aminoethyl ether)-N,N,N^1,N^1-tetraacetic acid (EGTA) and 5 parts serum (calcium 2.5 mM). The magnesium concentration of plasma is 1.0 mM. In medium SC6 with 5% heat-inactivated plasma the magnesium concentration is 0.7 mM. When making reduced magnesium culture medium (Mg^{2+}=0.15mM) 100 mM $MgCl_2$ concentrate is added to SC6, calcium free, magnesium free-5% heat-inactivated chicken plasma (culture medium with 5% heat-inactivated plasma contains 0.05 mM magnesium).

Agents Used

Protease inhibitors were dissolved in medium SC6, calcium free before being diluted into serum or plasma containing medium. Protease inhibitors once dissolved in SC6, calcium free were frozen (-20°C) until needed. Leupeptin and antipain were graciously provided by Dr. Walter Troll (New York University Medical Center) via the U.S.-Japan Co-operative Cancer Research Program. Heparin was obtained as a sterile solution, 10,000 USP unit per ml concentrate (Organon Ltd., Toronto). Epsilon-Aminocaproic Acid (EACA), Gamma-Aminobutyric-Acid (GABA), lysine HCl, benzamidine, TLCK (N-α-p-toxyl-L-lysine, Choloramethyl Ketone hydrochloride) were obtained from SIGMA. Soybean trypsin inhibitor and lima bean trypsin inhibitor were obtained from Worthington, Inc. (Freehold, N.J.) and Trasylol (pancreatic trypsin inhibi-

tor) from Mobay Chemical Corp. (New York, N.Y.). D-600 Hydrochlorid was obtained from Knoll AG Chemische Fabriken. N^2, $O^{2'}$-Dibutyryl Adenosine 3':5'-Cyclic Monophosphoric acid (Dbc 3', 5'-cyclic AMP), Adenosine 5'-Monophosphoric acid (5'-AMP) and EGTA were obtained from SIGMA. They were all dissolved in SC6-calcium free. Phorbol-12-myristate-13 acetate (PMA;SIGMA) and divalent cation ionophore A23187 (a gift from Eli Lilly Co. Indianapolis, Ind.) were dissolved in 99% ethanol. Human serum albumin was obtained from the Winnipeg Health Science Centre Chemistry Lab., and bovine serum albumin was obtained from SIGMA.

Cell Count Procedures

Test culture medium was aspirated off the dishes and 1.0 ml of complete count solution (Appendix D) was added to each dish. The dishes were put back into an isolette, for 1 hour on a rocker set for @1 cycle per minute, in the warm room (41.9°C). After 1 hour the dishes were brought out of the warm room, all the cells had been trypsinized and deattached from the culture surface. Then 2 drops of $CaCl_2$ 20 x (Appendix D) and 2 drops clarified calf serum were added to each dish to prevent cell destruction during trituration.

The culture dish medium in each dish was then triturated 21 times with a Pasteur pipet to wash off any remaining cells from the culture dish surface. This triturated (1 ml) medium was transferred to a accuvette (Coulter Electronics, Inc., Hialeah, Fla.) containing 8.9 mls of Isoton solution (Coulter Electronics, Inc.) and then the cells were counted on a Coulter Model ZF Electronic Cell Counter using a 100 uM aperture. The following

settings were used to get the best display and counts on the oscilloscope screen, amplication setting at 1, aperature current 1/8 mAMPS and threshhold at 6. The Coulter counter was set at the above settings to allow cell counts only, not debris. After the counts were read they were corrected using a coincidence correction chart, issued by Coulter Counter Electronics.

For growth curves, each experimental point represents the mean $\pm$ SEM (Standard Error of Mean) of 2 culture dishes. The biometrics were done on a SR-52 programable calculator.

Photography

Photographs of the cell cultures were taken using a NIKON 35 mm camera M-35S (Nippon, Kogku, New York) with an AFMB attachment. The camera and AFMB attachments were mounted on a Leitz Wetzlar Diavert inverted phase contrast microscope (Ernst Leitz Ltd., Ontario). Black and white pictures were taken with Kodak Tri-X pan film (135-36). Photograph developing was done by Erika Jahnke, pathology department.

RESULTS

RESULTS

Description of the Medium Change Response

Normal chicken fibroblasts have a fan shape and form a relatively "regular" growth pattern in cell culture (Figures A, B). Chicken fibroblasts infected with the Schmidt-Ruppin strain of the Rous sarcoma virus have a spindle shape and form an "irregular" growth pattern in culture, such as growing on top of each other forming "criss-crosses" (Figures C, D). Within 5 minutes after change to fresh chicken serum or heat-inactivated chicken plasma-containing medium, most RSV-infected fibroblasts in a culture assume a round shape, detach partially or completely from the culture surface in moderate or high density cultures and form large aggregates (Figures E, F). Immediately after the medium change the RSV-infected fibroblasts undergo blebbing of the plasma membrane prior to rounding (Figures G,H). In the case of moderate or high density cultures, where cellular aggregates form, aggregation closely follows cellular rounding and detachment. The aggregates formed appear to involve retraction of portions of the cell monolayer (Figure E). This series of changes reaches completion within five minutes, as noted above, and proceeds at room temperature or in the cell culture incubator. I have called the cellular rounding, detachment and aggregation that follow medium change the "medium change response" (MCR).

The MCR is not observed in control cultures of "normal" (non-infected) chicken fibroblasts. Normal fibroblasts have

been very carefully observed immediately after fresh medium change and up to several hours after. I have developed a grading system determining the strength of the MCR, involving a visual analysis of the MCR on RSV-infected fibroblasts undergoing MCR and subsequent consequences of the response. Figures I, J, K, L show a description of the strengths of the MCR that may occur.

The magnitude of the MCR was graded on a 0 to 4 scale. Zero refers to no MCR. A MCR of "1" involves few cells (~25%) rounding up with no dramatic change of the monolayer (Figure I). A MCR of "2" involves few to many cells (~50%)rounding up with changes to the monolayer such as formation of small gaps in the monolayer, and cells rounding and forming aggregates (Figure J). A MCR of "3" involves many cells (~75%) rounding, formation of large aggregates, retraction of the monolayer at certain areas and formation of large gaps in the monolayer (Figure K). Finally, the highest strength MCR is "4", which is when most cells round up and the monolayer is completely disrupted and consists of large and small aggregates of rounded up cells (Figure L).

After the MCR has occurred, essentially all RSV-infected fibroblasts in low density cultures re-attach to the culture dish surface and spread partially within 1 hour and completely within 2 hours. Failure of a fraction of infected cells to emerge from aggregates appears to be responsible for the ultimate presence of cell clumps in high density cultures of RSV-infected fibroblasts that have been subjected to daily medium changes over a 4-5 day period (Figures M, N, O, P). The presence of such clumps

in high density cultures is generally considered to be a characteristic of Rous sarcoma virus infection (25). No cell clumps are present in high density cultures of normal fibroblasts (Figure 0).

The MCR is observed in RSV-infected fibroblasts usually after 3 days of infection as secondary cultures. As tertiary and quartenary cultures the RSV-infected fibroblasts give a strong MCR. Experimental trials were with tertiary cultures since the results were similar using both tertiary or quartenary cultures. The virus infection can be observed when normal cells adopt a spindle shape, usually occurring within two days of infection; the MCR follows a day or two later. Visible observation along with proliferative rates is used to determine the extent of infection.

The possibility of the MCR occurring only on Lux Contur dishes was examined. Falcon culture dishes were used and were found to give results identical to the Lux Contur dishes.

Activities of Plasma, Sera from Various Species (Table 1, 2)

Chicken plasma and chicken serum (5%) are equally effective in inducing the MCR. The MCR occurs when fresh chicken plasma or serum is added to unchanged cultures as well as occurring when these cultures are changed to complete media. Heat-inactivation of chicken plasma or serum does not alter their ability to induce a MCR. In addition to chicken serum or plasma, a full-strength MCR is induced by human adult or umbilical cord serum, bovine calf serum and fetal bovine serum.

There is no difference between commercial chicken serum and freshly prepared chicken serum in producing a MCR. At the same time there is no difference between freshly made heat-inactivated chicken plasma and freshly made chicken serum in producing a MCR.

The MCR does not occur when spent (incubated on cell cultures) chicken plasma or serum containing medium is aspirated from and then returned to another RSV-infected cell culture as a medium change (Table 3) or when spent medium is replaced with serum-free synthetic medium; these results indicate the MCR does not have a physical basis and that the MCR does not occur in response to a crystalloid present in the synthetic medium. Addition of albumin, in synthetic culture medium, does not induce the MCR.

The MCR occurs at full strength with medium containing chicken serum at concentrations as low as 2% and is still observed, although at reduced strength, with serum concentrations as low as 0.5% or 0.25% (Graph 1). At these low concentrations the MCR is reduced and the reaction time is slower (4-8 minutes) compared to the faster reaction at higher concentrations.

Addition of medium SC6 with 5% serum of clarified chicken plasma on a culture medium change produces a MCR of "4" (Table 2). RSV-infected fibroblasts grown in medium SC6 with 5% serum of clarified chicken plasma show no reduction of the MCR. These results indicate that the MCR does not likely involve one of the blood coagulation cascade factors. Most of the major factors would be used up in the exhaustive clotting of the plasma.

Loss of Chicken Serum or Heat-Inactivated Chicken Plasma Culture Medium Ability to Induce MCR (Table 3, Graph 2)

After 5 hours of exposure to moderately dense cultures of RSV-infected fibroblasts or "normal" fibroblasts, culture medium containing 5% serum will no longer induce the MCR when transferred to other dishes of RSV-infected fibroblasts. Serum-containing medium that is incubated in culture dishes without cells 5 or 24 hours shows no diminution in capacity to induce the MCR when added to RSV cell cultures. These observations suggest that the serum component(s) that induces the MCR is inactivated or consumed by both cell types during the period of incubation.

Transfer of medium, 3 to 10 minutes after medium change, from RSV-infected cultures to cultures of "normal" fibroblasts does not result in detachment, rounding or aggregation of the normal fibroblasts. These cultures upon addition of RSV culture medium after several hours showed no indication of a MCR.

Activities of Serum Ultrafiltrates and Dialyzed Serum (Table 4)

The MCR is not induced by ultrafiltrates of chicken or human serum prepared with a molecular weight cutoff level of approximately 50,000 daltons. This ultrafiltrate was added to cultures undiluted as well as at a concentration of 5% in synthetic medium. In addition, no MCR was observed when the 50,000 dalton cutoff ultrafiltrate was added to spent complete medium at a final concentration of 5%. The retentate was added in undiluted form and in a concentration of 5% in synthetic medium to cultures, producing a MCR of "4".

The MCR is induced by ultrafiltrates, both diluted and undiluted (5%), of chicken and human serum prepared with a molecular weight cutoff level of approximately 100,000 daltons. A full MCR was induced by the undiluted ultrafiltrate indicating that induction of the MCR involves a serum (plasma) macromolecule(s) in the approximate molecular weight range of 50,000-100,000 daltons. Also this ultrafiltrate was added to spent culture medium and produced a strong MCR. The MCR occurs upon addition of exhaustively, dialyzed chicken serum, suggesting that the inducer is the 50,000-100,000 dalton macromolecule(s) itself, rather than a small molecular weight molecule bound to the macromolecule.

Heat Stability of the MCR Inducer Macromolecule(s) (Table 5)

Chicken serum was boiled for 1 hour and added to cultures in diluted and undiluted form. The undiluted, boiled serum gave a full strength MCR while the diluted (5% boiled serum in medium SC6) gave a MCR of "2". Chicken and human serum were boiled for 2 hours and added diluted and undiluted (5%) to cultures. The chicken serum boiled 2 hours gave identical results as when boiled for 1 hour. The 2 hour boiled human serum diluted and undiluted gave a full strength MCR indicating the inducer macromolecule(s) has considerable heat stability, perhaps more so than the chicken serum. Precipitated protein molecules of boiled serum may protect the denaturing of other protein molecules so possibly the MCR inducer macromolecule(s) could have been protected by these precipitated proteins.

Activities of Plasminogen and Cells Grown in Deplasminogenated Serum (Table 2)

The MCR does not occur upon addition of chicken plasminogen in an amount equivalent to that present in medium containing 25% chicken serum. Chicken plasminogen (25% serum equivalent) was added to spent culture medium, but after observations for 1 hour, no MCR was detected. The MCR occurs, at full strength, when deplasminogenated chicken serum was added to cultures of RSV-infected fibroblasts, regardless of whether the RSV-infected cells had been cultivated in standard medium or had been cultivated, ab initio, in medium containing plasminogen-free chicken serum. Normal fibroblasts were cultivated as secondary cells in plasminogen-free chicken serum, infected with plasminogen-free chicken serum Rous sarcoma virus inoculum and subcultured to tertiary cultures in plasminogen-free chicken serum culture medium. These observations with plasminogen-free serum and plasminogen indicate that plasminogen is not the inducer of the MCR and that the MCR does not involve activation of plasminogen and does not involve plasmin.

Effect of Protease Inhibitors and Heparin on the MCR (Tables 6, 7, 8)

Protease inhibitors and other agents in culture medium were added to tertiary RSV-infected fibroblast cultures on the third day after passage. On the third day the cultures are always subconfluent, flat with few clumps and a good strong MCR occurs upon addition of chicken serum or heat-inactivated chicken plasma containing culture medium. On day 0 and 2 there are not enough cells to obtain a good evaluation of the MCR, while on day 4 most of the cells are in clumps.

The MCR ceased to occur after RSV-infected fibroblasts had been cultivated for 3 days in medium that contained 10^{-5}M leupeptin or 2 units/ml heparin (Table 6). Concentrations of protease inhibitors had to be found that would inhibit the MCR but at the same time not reduce the cells proliferative rate (Graphs 3, 4). Antipain could prevent the MCR but would decrease the proliferative rate of the cells. Partial abolition of the MCR was observed when RSV-infected fibroblasts were cultured in the presence of 10^{-6}M leupeptin, 10^{-5}M, 10^{-6}M antipain, at 100 mM or lower concentrations of FACA and GABA, 100 ug/ml soybean trypsin inhibitor or 1000 KIU/ml Travsylol (Table 6). In trials were the MCR was partially inhibited after the cells had been cultivated for several days in an inhibitor, a hedging pattern of the cells occurred. Blebbing was often more pronounced, as though the cells were trying to produce a MCR but were prevented after the blebbing stage.

The RSV-infected fibroblasts showed similar MCR when cultivated in chicken serum or heat-inactivated chicken plasma culture medium containing these protease inhibitors (Tables 6, 7). Lysine only partially inhibited the MCR at 10 mM. Higher concentrations were toxic and lower concentrations did not inhibit the MCR.

In presence of 10^{-5}M leupeptin or 2 units/ml heparin, high density cultures of RSV-infected cells manifest a flat, rather than a clumped appearance on day 2, 3 and 4 (Figures R,S). The morphology of individual RSV-infected cells was not, however, affected by either of these agents. The MCR does not occur when cells which have been cultivated in the presence of leupep-

tin or heparin, were washed and exposed to fresh medium without either agent.

Neither leupeptin, at concentrations up to 10^{-3}M, nor heparin, at concentrations up to 1000 units/ml were capable of abolishing the MCR when added to cultures for the first time with fresh chicken serum or heat-inactivated chicken plasma containing medium (Table 8). Leupeptin and heparin at the above concentration were added to serum containing medium 5 days before the medium was used; this medium produced a full-strength MCR. This finding indicates that these agents do not act by binding slowly to any serum component but, rather, that they act on the cultured RSV-infected fibroblasts themselves. Leupeptin, heparin and EACA are serine protease inhibitors, suggesting that a serine protease may be involved in the MCR. TLCK, a trypsin titrant, was dissolved in serum containing culture medium and added to RSV-infected fibroblasts, the MCR was not reduced. Cells were not grown out in medium containing TLCK due to the unstable and toxic characteristics of this agent.

Amphojel ($Al(OH)_3$) was added to heat-inactivated plasma to absorb the serine proteases prothrombin, factor X, factor IX and factor VII present in the plasma, however, a full strength MCR occurred after absorption (Tables 2, 7). RSV cultures grown in absorbed heat-inactivated plasma showed no dimunition in the MCR.

RSV-infected fibroblasts seeded in culture medium containing 5% heparinized (40 units/ml) chicken plasma and cultured for a 4-day period showed similar MCR overall, compared to cells which were seeded in SC6-5% heat-inactivated chicken plasma and

changed to SC6-5% heparinized (40 units/ml) chicken plasma. Seeding cells with other protease inhibitors affected overall growth rates slightly. This indicates that the cells are very sensitive at the seeding stage and cannot cope with the addition of protease inhibitors.

Effect of Protease Inhibitors and Heparin on Cell Growth (Graphs 3, 4)

RSV-infected fibroblast's proliferative rates were not affected in the presence of 10^{-5}M leupeptin or 2 units/ml heparin in medium SC6 with 5% chicken serum when cultivated for a period of 4 days (Graph 3). These cells were seeded in medium SC6 with 5% chicken serum and changed on day 0 to medium containing the protease inhibitor. Medium SC6 with 5% chicken serum was the control. The control trial had a slightly reduced final count of cells, this is most likely due to the loss of unattached cells during medium aspiration for cell counting. The cultures cultivated with protease inhibitors were firmly attached to the dish.

Proliferative rates of RSV-infected fibroblasts were not affected in the presence of 10^{-5}M leupeptin in medium SC6 with 5% heat-inactivated chicken plasma (Graph 4). The cells were seeded in medium SC6 with 5% heat-inactivated chicken plasma and changed on day 0 to medium SC6 with 5% heat-inactivated chicken serum or medium SC6 with 5% heat-inactivated chicken plasma, 10^{-5} leupeptin.

Effect of Calcium on the MCR (Tables 9, 10)

A markedly reduced MCR was observed with the addition of serum containing medium of reduced (0.125mM) calcium concentra-

tion (Table 10). The reduced MCR was observed throughout a 4-day period of cultivation of RSV-infected fibroblasts in medium with reduced calcium medium (Table 9). A slight reduction in strength of the MCR was observed when media of very low ($\sim 10^{-5}$M) calcium concentration was added for the first time. This varied, however, perhaps due to the ability of EGTA to chelate calcium in the culture medium. The culture medium was prepared with EGTA a day before use. The cells were washed twice with PEG-CF to remove unbound calcium from the cell surfaces.

No reduction of the MCR occurred when magnesium was reduced (Tables 9, 10).

When divalent cation ionophore A23187 was added, at concentrations of 10^{-5}M and 10^{-6}M no MCR was induced. (The cells were observed for 30 minutes after addition of ionophore). The ionophore was used to determine if the MCR involved an increase of calcium ion in the cell cytosol.

D-600, a calcium antagonist, at 10^{-4}M did not reduce the MCR upon fresh medium change (Table 10). RSV-infected fibroblasts cultivated in medium SC6 with 5% heat-inactivated plasma, 10^{-4}M D-600 showed no reduction in MCR. D-600 was used to determine if the MCR depends on calcium influx into the cell.

Effect of Other Agents on the MCR

RSV-infected cells were cultivated with Dibutyryl 3', 5' cyclic AMP and 5'-AMP in serum containing medium for a period of 4 days. No MCR reduction was observed during this period of cultivatin (Table 11). Dibutyryl 3', 5'-cyclic AMP in medium SC6 was added to RSV-infected fibroblasts as fresh

medium to induce the MCR, however, no MCR was observed (Table 2). M. Weber reported that addition of Dibutyryl 3', 5'-cyclic AMP to cultures of RSV-infected chick embryo fibroblasts increases their adherence (101). In my results no increase of adherence of RSV-infected chicken fibroblasts was detected. Dibutyryl 3', 5'-cyclic AMP in medium SC6 added to RSV-infected fibroblasts for the first time did not produce a MCR (observed for 1 hour). Dibutyryl 3', 5'-cyclic AMP had been reported to cause Y-1 cells (mouse adrenalcortical-tumor cell line) to round up (111).

Effects of Phorbol-12-Myristate-13 Acetate on Cells

Secondary normal fibroblasts were cultivated in chicken serum containing medium with 5 ng/ml phorbol-12-myristate-13-acetate (PMA). By day 2 some of these cells formed a spindle-shaped appearance similar to RSV-infected fibroblasts. On day 5 these cells were passaged in SC6-5% chicken serum, 5 ng/ml PMA, and were then cultured in chickem serum containing medium with 5, 10, and 50 ng/ml PMA as tertiary cells and later as quartenary cells. More of the cells in 5 ng/ml PMA became spindle shaped (Figure T). Cells in 10 and 50 ng/ml PMA became elongated with a spindle shape identical to RSV-infected fibroblasts. Growth was reduced in the 10 and 50 ng/ml PMA cultures. These cells were cultivated with the various PMA concentrations as tertiary and quartenary cells, however, none of the cells manifested a MCR.

Effects of Continuous Medium Changes on RSV-Infected Fibroblasts (Table 12, Graph 5)

An attempt was made to mimic the *in vivo* situation by

changing cultures of RSV-infected fibroblasts to fresh medium at short intervals after they had sustained an initial MCR (Table 12, Graph 5). When the culture medium was changed every hour after the initial medium change, no MCR occurs until the 5th and 6th hour (a MCR of 1/2; Table 12).

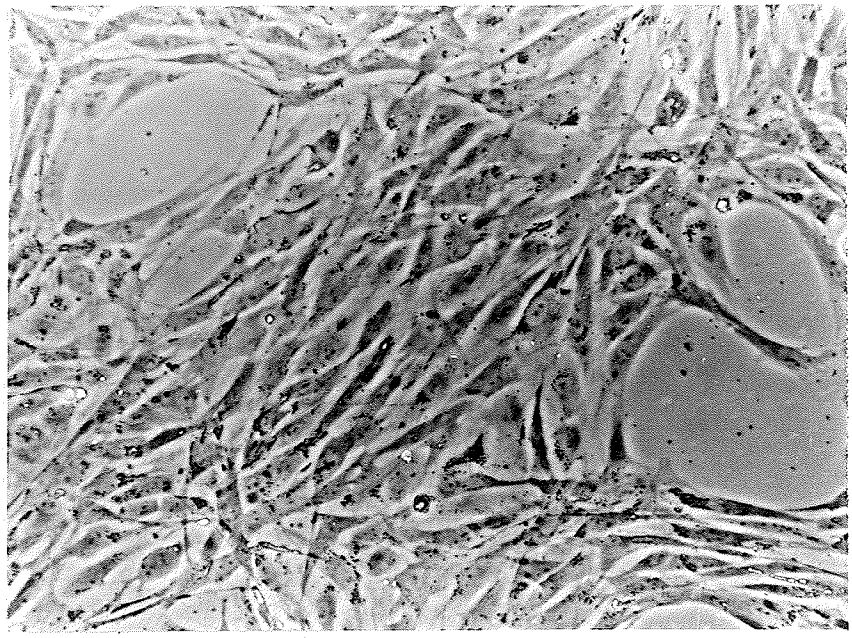
Culture medium that was changed every 2 hours after the initial medium change gave a MCR of "1" after 2 hours and a MCR of 1/2 after 4 hours. Culture medium changed on the 5th hour after the initial medium change gave a MCR of 4. There seems to be a 4-hour regeneration period before the cells will produce a MCR (Graph 5). These observations show that the MCR is abrupt and accentuated when medium changes were made in intervals of 5 hours but occurs at a low continuous level when medium was changed often (every 1 or 2 hours).

pH of Culture Medium Containing Serum or Heat-Inactivated Plasma

The pH of culture medium containing serum or plasma inactivated before being put on the cell cultures was @pH 7.4 (physiological pH standard). After the addition of this medium to RSV-infected fibroblasts for 5 minutes there was no change in pH. Complete culture medium left on moderate or high density RSV-infected fibroblasts for a day will become slightly acidic, a pH of @6.8. Spent complete culture medium (6-hour incubation of SC6-5% heat-inactivated chicken plasma on moderate density RSV-infected fibroblasts) was found to have a pH of approximately 7.3. Spent complete culture medium (24 hours incubation) with a pH @ 6.8 was raised to pH 7.4 by the addition of 1 N NaOH and added to the RSV-cultures. No MCR occurred.

Figure A. Subconfluent normal control chicken fibroblasts in cell culture on day 2. Cells were grown in medium SC6 with 5% heat-inactivated chicken plasma. The cells have a fan shape and a relatively "regular" growth pattern (Cells are flat, arranged in a parallel orientation). Phase contrast X100.

Figure B. Subconfluent normal control chicken fibroblasts in cell culture on day 2. Cells were grown in medium SC6 with 5% heat-inactivated chicken plasma. The fan shape of the cells can be seen distinctly. The swirling pattern is a characteristic of a "regular" growth pattern of normal control chicken fibroblasts. Phase contrast X200.



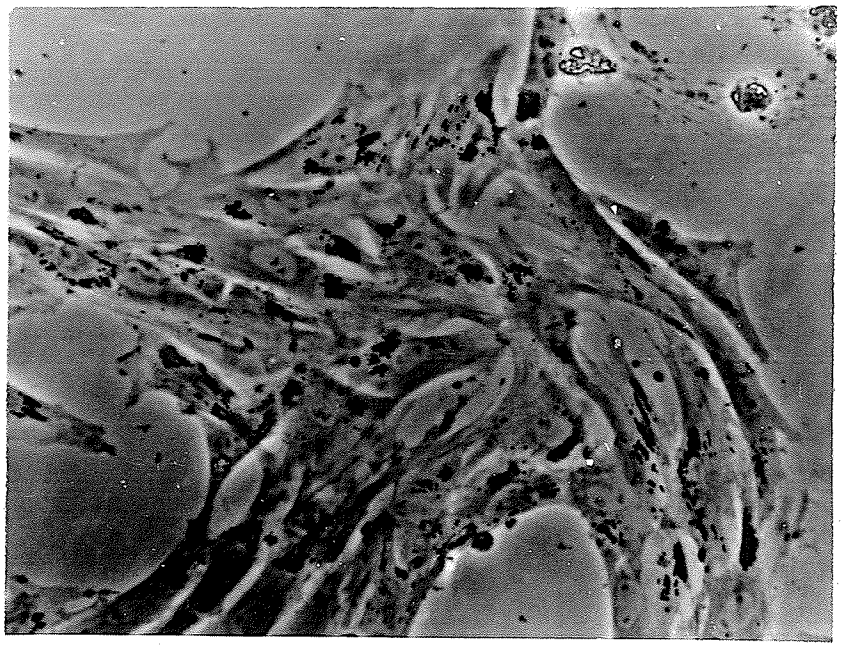
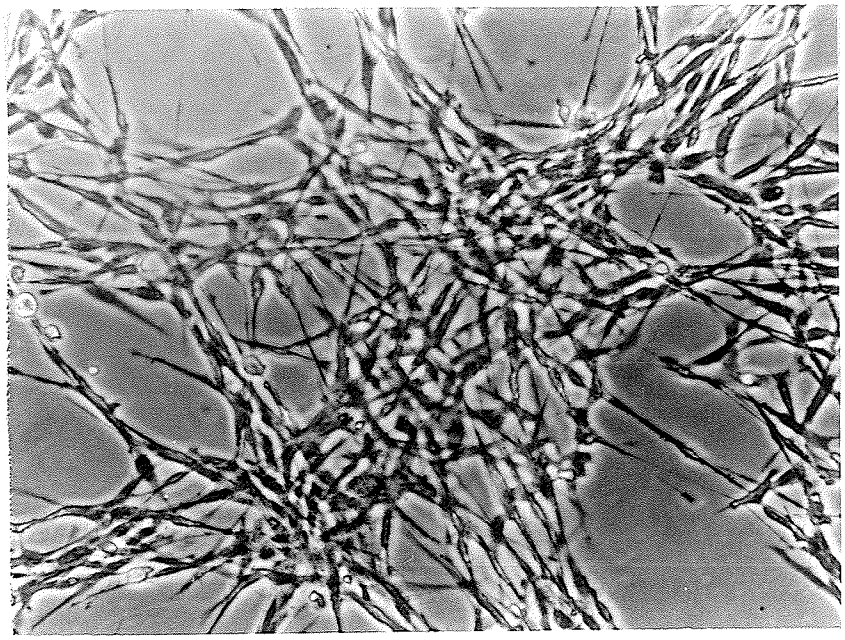


Figure C. Subconfluent Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 2. Cells were grown in medium SC6 with 5% heat-inactivated chicken plasma. The cells have a narrow spindle shape with an "irregular" growth pattern (Cells grow on top of each other forming a criss-cross pattern). Phase contrast X100.

Figure D. Subconfluent Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 2. Cells were grown in SC6 with 5% heat-inactivated chicken plasma. The cells narrow spindle shape can be seen. The criss-cross "irregular" growth pattern is distinct. These RSV-infected cells are also present in the shape of a single or double pyramid (top right corner). Phase contrast X200.



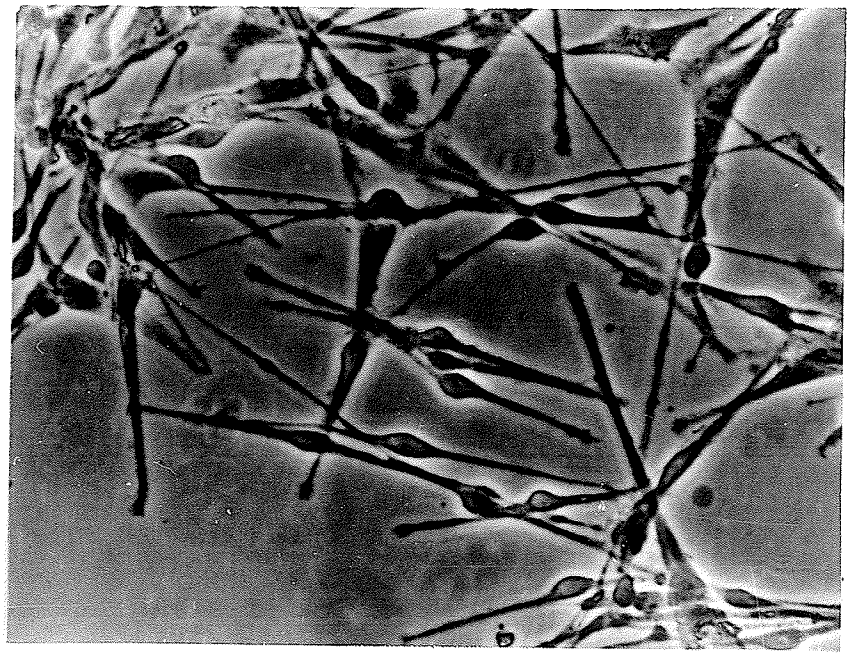
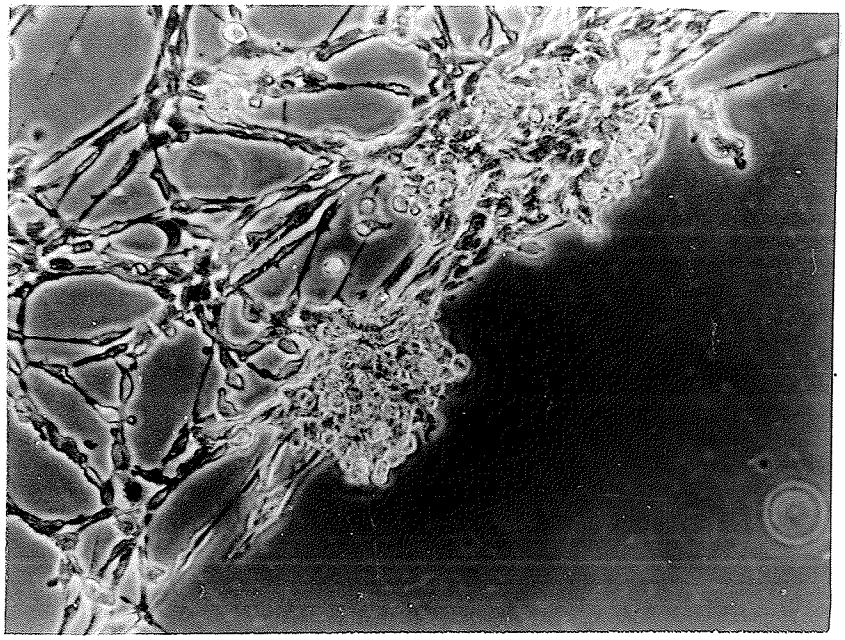


Figure E. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 3. Cells were grown in medium SC6 with 5% chicken serum. On addition of fresh chicken serum-containing medium the RSV-infected fibroblasts assume a round shape or shrink in size and detach partially or completely from the culture surface. Aggregation follows cellular rounding and detachment. In the figure retraction of portions of the cell monolayer can be seen. Phase contrast X100.

Figure F. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 3. Cells were grown in medium SC6 with 5% heat-inactivated chicken plasma. On addition of fresh chicken serum-containing medium the RSV-infected fibroblasts round up and detach forming aggregates. Phase contrast X100.



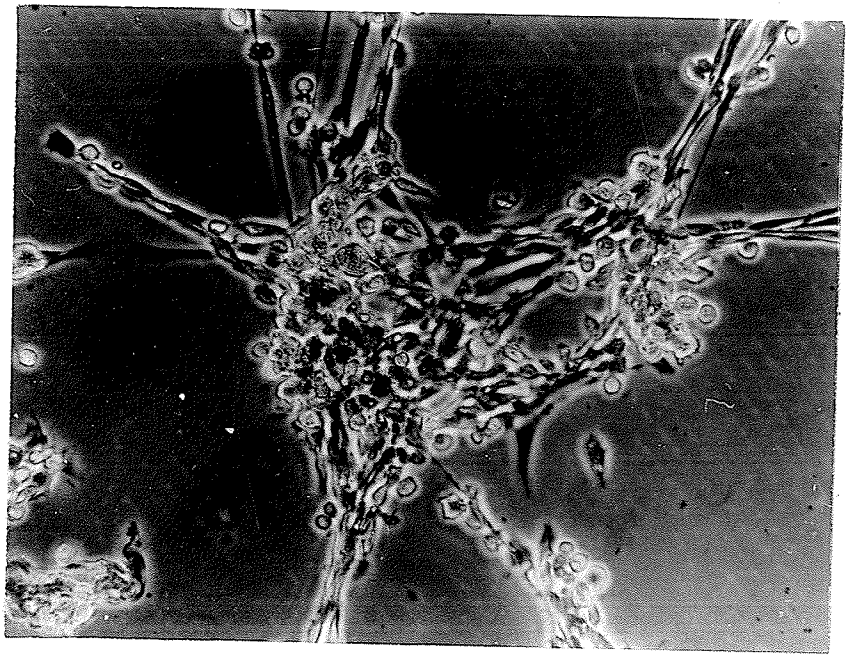
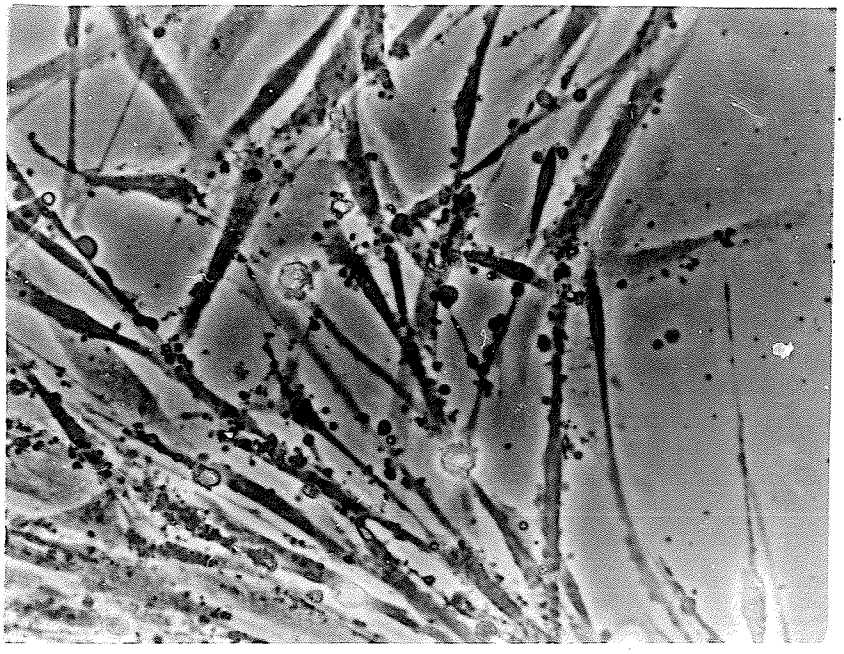


Figure G. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 3. Cells were grown in medium SC6 with 5% heat-inactivated chicken plasma. Immediately after addition of fresh chicken serum-containing medium the RSV-infected fibroblasts undergo blebbing of the plasma membrane prior to rounding. Round fragments can be seen detached from the blebbed RSV-infected fibroblasts. Phase contrast X200.

Figure H. As figure G. Phase contrast X200.



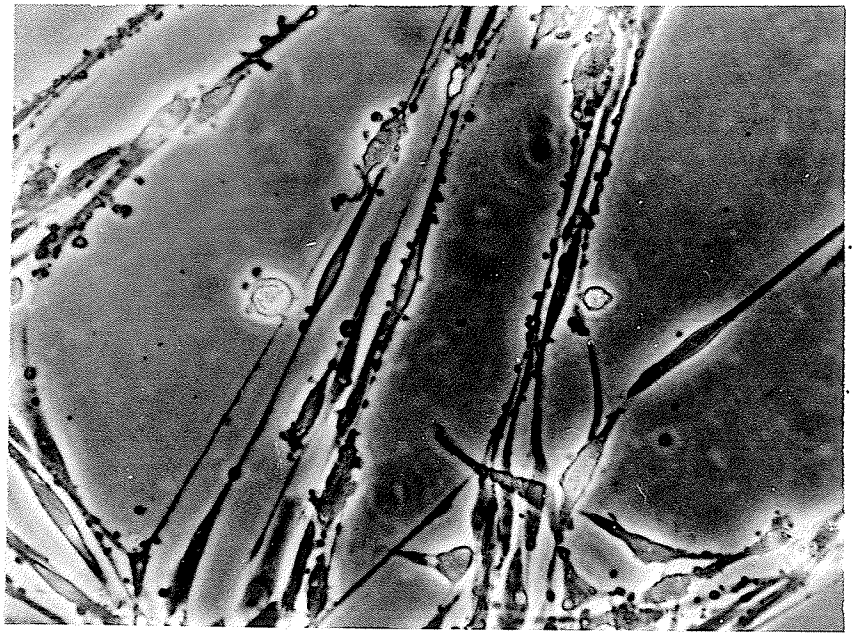
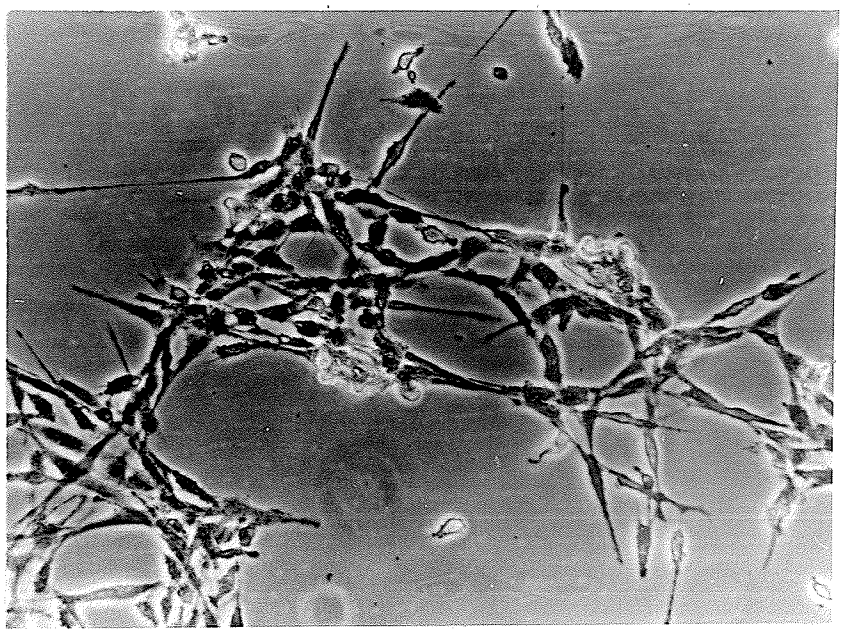


Figure I. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 3. Cells were grown in medium SC6 with 5% chicken serum. On addition of fresh chicken serum-containing medium the RSV-infected fibroblasts undergo a MCR of "1" (A few cells round up, small aggregates are present but there is no dramatic change of the monolayer). Phase contrast X100.

Figure J. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 3. Cells were grown in medium SC6 with 5% chicken serum. On addition of fresh chicken serum-containing medium the RSV-infected fibroblasts undergo a MCR of "2". (Few to many cells round up. Changes in the monolayer occur such as formation of small gaps with many aggregates of cells). Phase contrast X100.



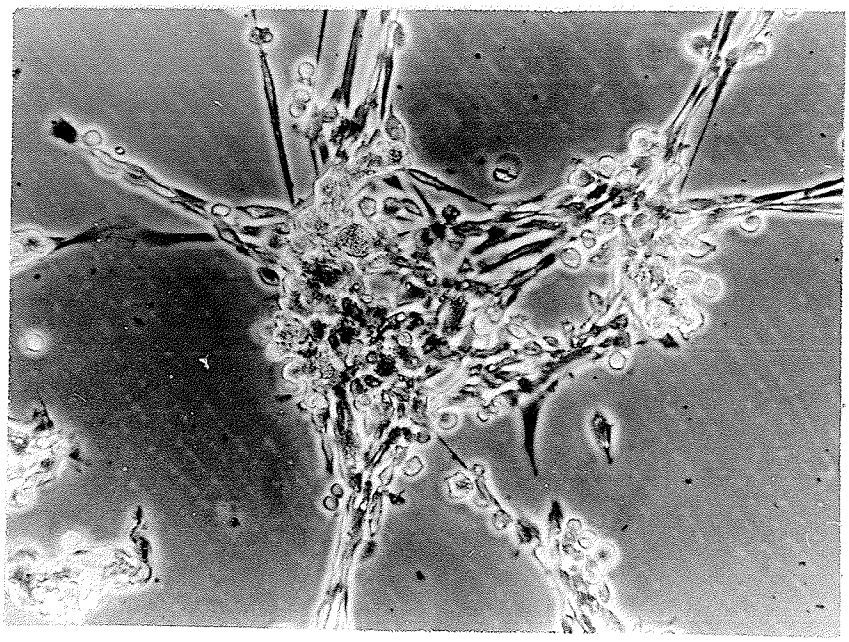
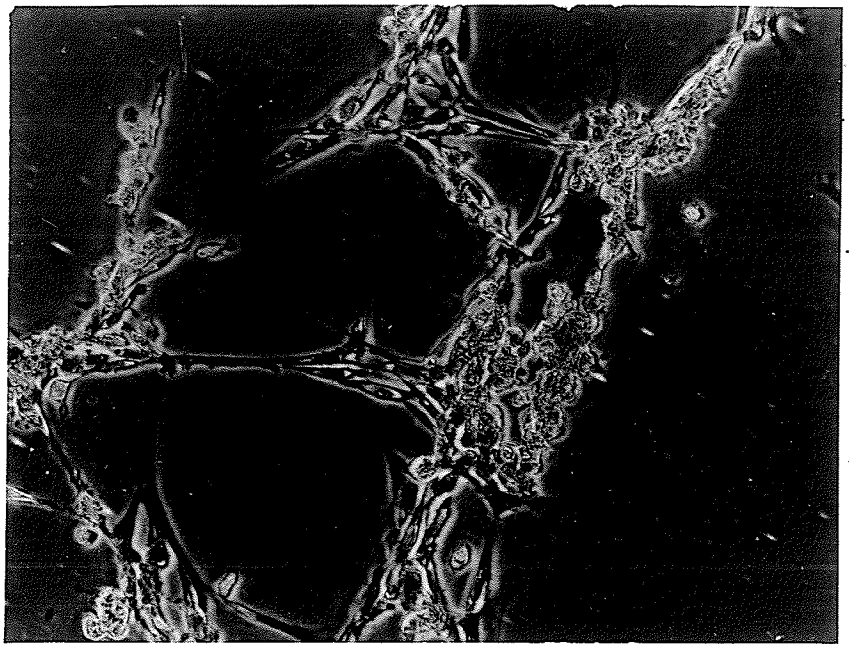
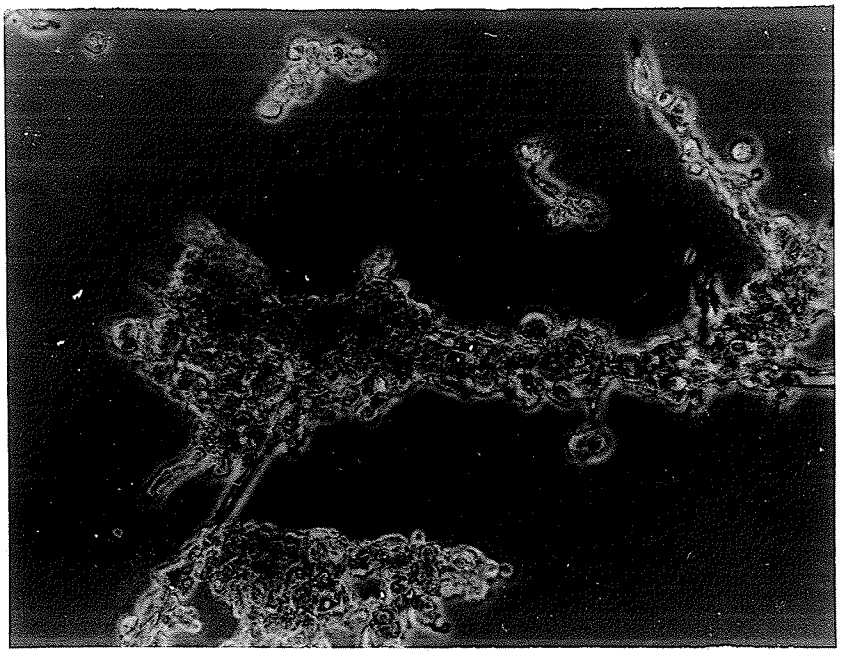
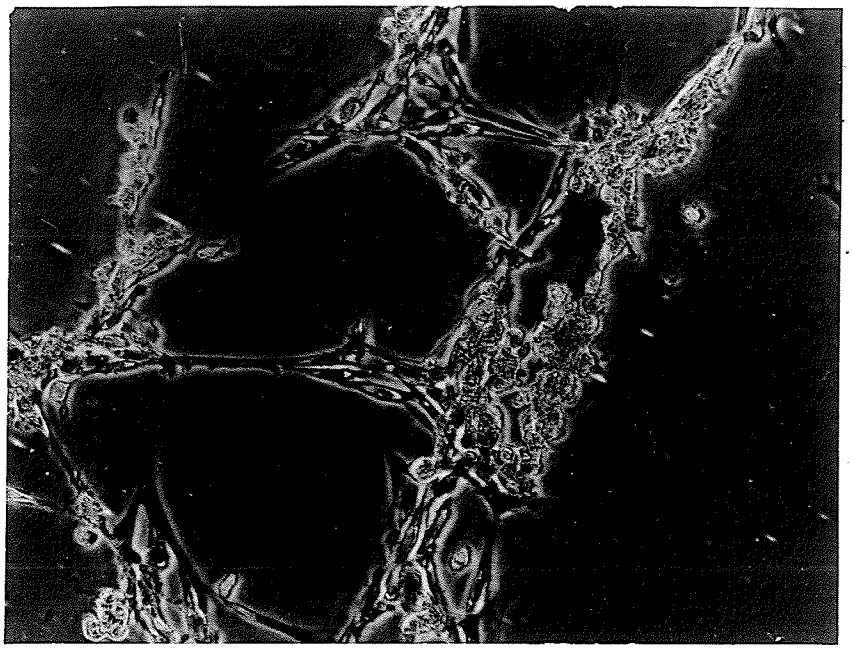


Figure K. Rous sarcoma virus-infected fibroblasts in cell culture on day 3. Cells were grown in medium SC6 with 5% chicken serum. On addition of fresh chicken serum-containing medium the RSV-infected fibroblasts undergo a MCR of "3" (Many cells round up, many large aggregates are formed, portions of the monolayer retract, large gaps form in the monolayer and cell aggregates detach from the culture surface). Phase contrast X100.

Figure L. Rous sarcoma virus-infected fibroblasts in cell culture on day 3. Cells grown in medium SC6 with 5% chicken serum. On addition of fresh chicken serum-containing medium the RSV-infected fibroblasts undergo a MCR of "4" (Most cells have rounded up, the monolayer is completely disrupted, and large and small aggregates of cells detach from the culture surface). Phase contrast X100.







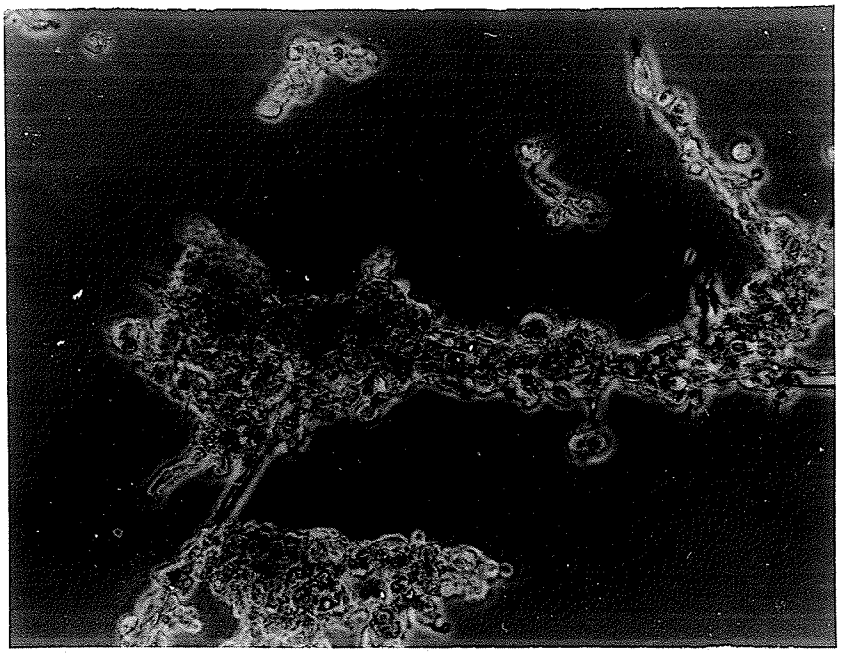
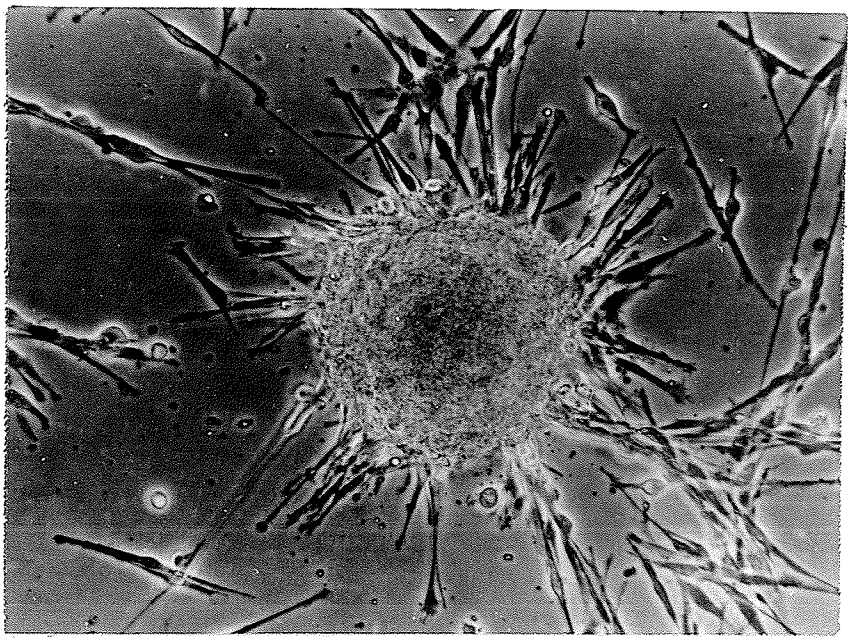


Figure M. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 4. Cells were grown in medium SC6 with 5% chicken serum. There is a large aggregate of rounded cells in a clump. Spindle-shape cells can be seen projecting out from the edge. This is a typical example of high density RSV-infected fibroblast culture in SC6-5% chicken serum. Phase contrast X100.

Figure N. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 4. Cells were grown in medium SC6 with 5% chicken serum. On addition of fresh chicken serum-containing medium spindle-shape cells on the edge of the clumps retract. Blebbing can be seen on some of the cells. Phase contrast X100.



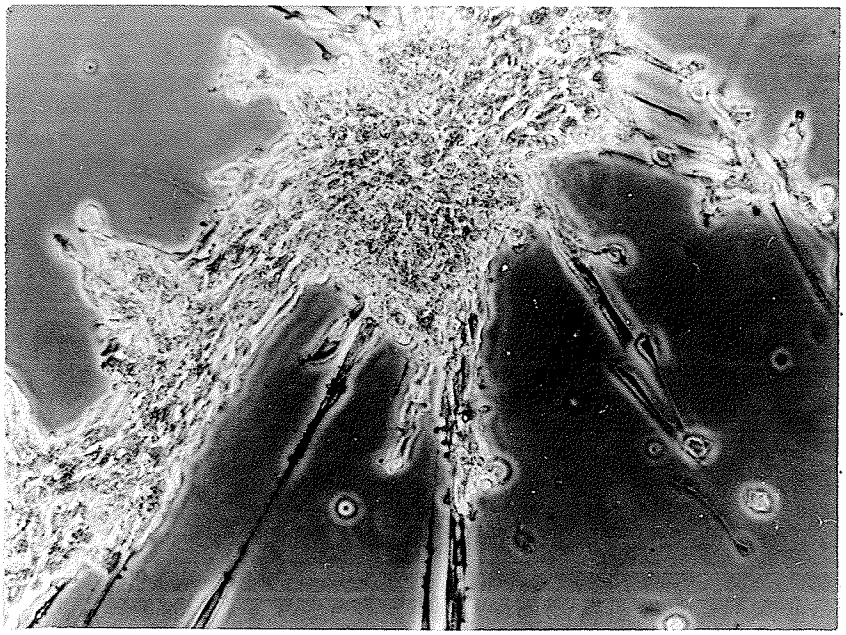
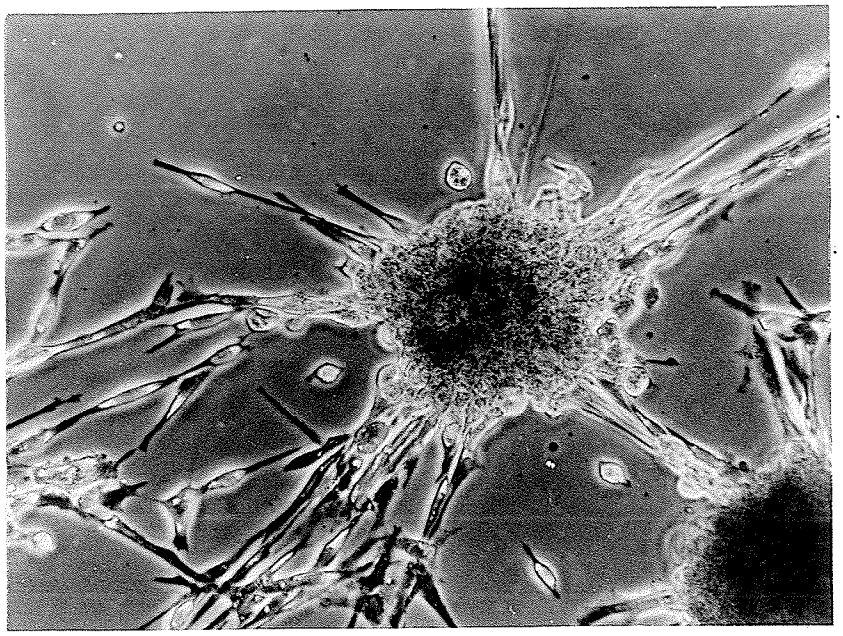


Figure O. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 4. Cells were grown in medium SC6 with 5% chicken serum. Two large aggregates of rounded cells with spindle-shape cells projecting out can be seen. Phase contrast X100.

Figure P. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 4. Cells were grown in medium SC6 with 5% chicken plasma inactivated. Several small aggregates of rounded cells can be seen. Spindle-shape cells project out giving a stellate appearance. Phase contrast X100.



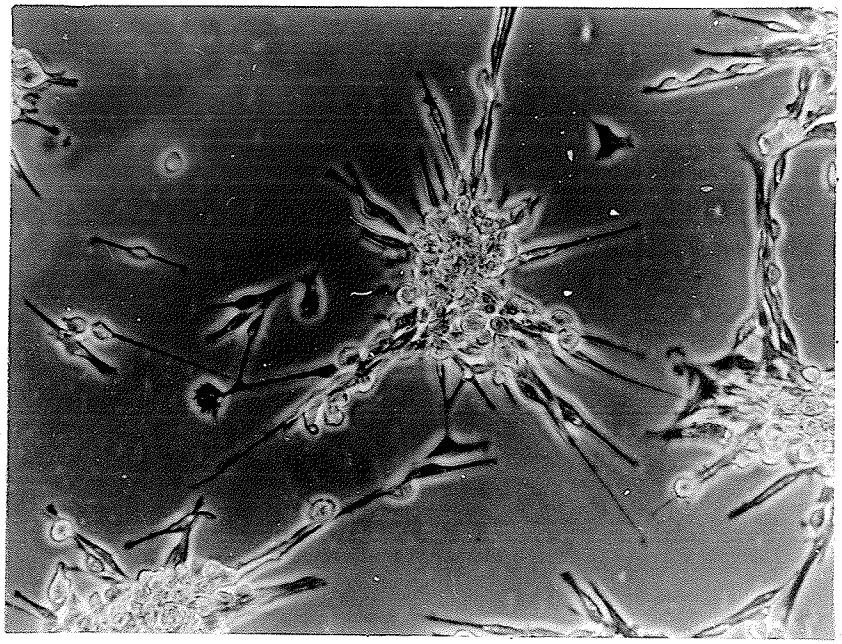


Figure Q. Normal control chicken fibroblasts in cell culture on day 4. Cells were grown in medium SC6 with 5% heat-inactivated chicken plasma. A flat monolayer of fibroblasts with a parallel orientation is seen in this typical normal control high density culture. Phase contrast X100.

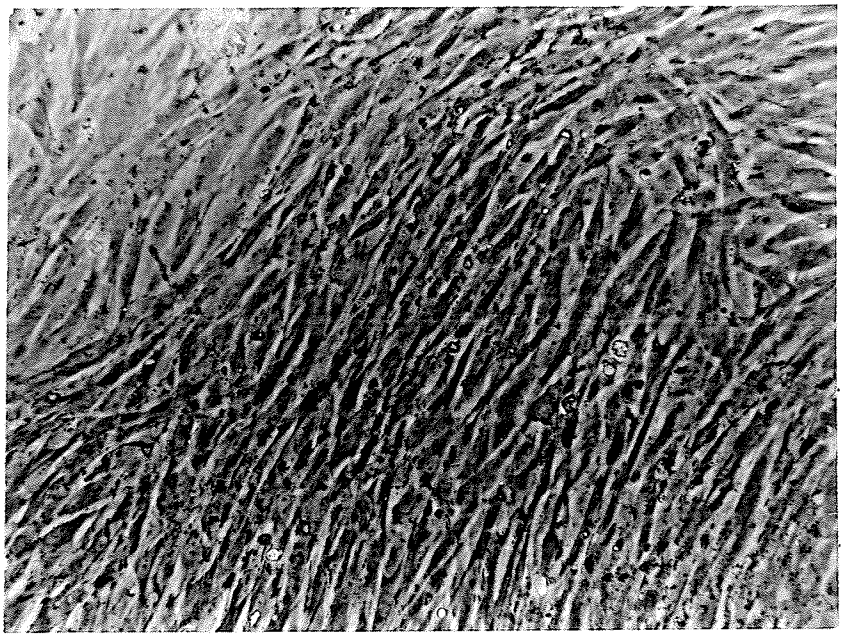
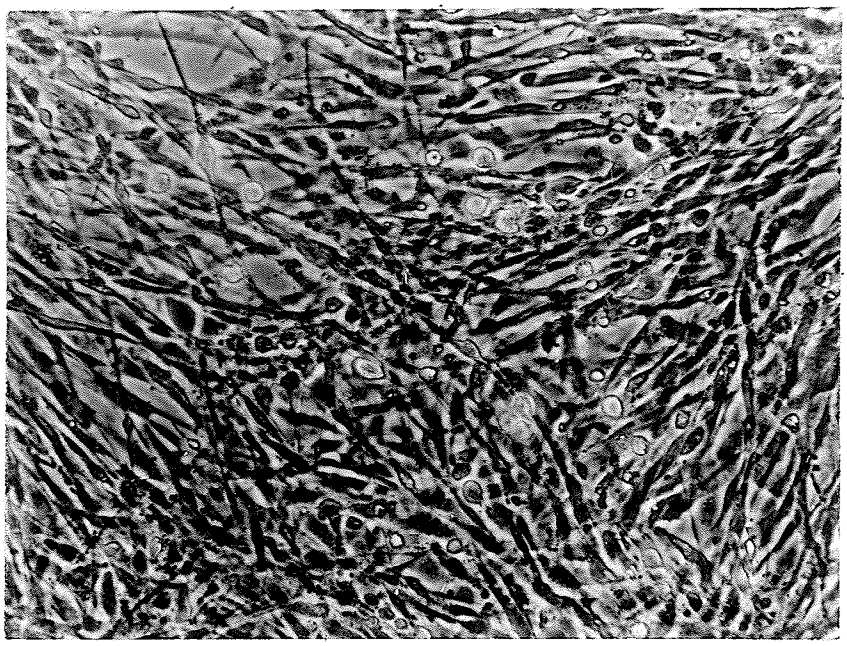


Figure R. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 4. Cells were grown in medium SC6 with 5% chicken serum, 2 units/ml heparin. A flat monolayer of cells is seen in this high density culture of RSV-infected fibroblasts. The cells have a spindle shape and form an "irregular" criss-cross pattern. Phase contrast X100.

Figure S. Rous sarcoma virus-infected chicken fibroblasts in cell culture on day 4. Cells were grown in medium SC6 with 5% chicken serum, 10^{-5}M leupeptin. A flat monolayer of cells is seen in this high density culture of RSV-infected fibroblasts. The shape and pattern of growth of the cells is similar to figure R. Phase contrast X100.



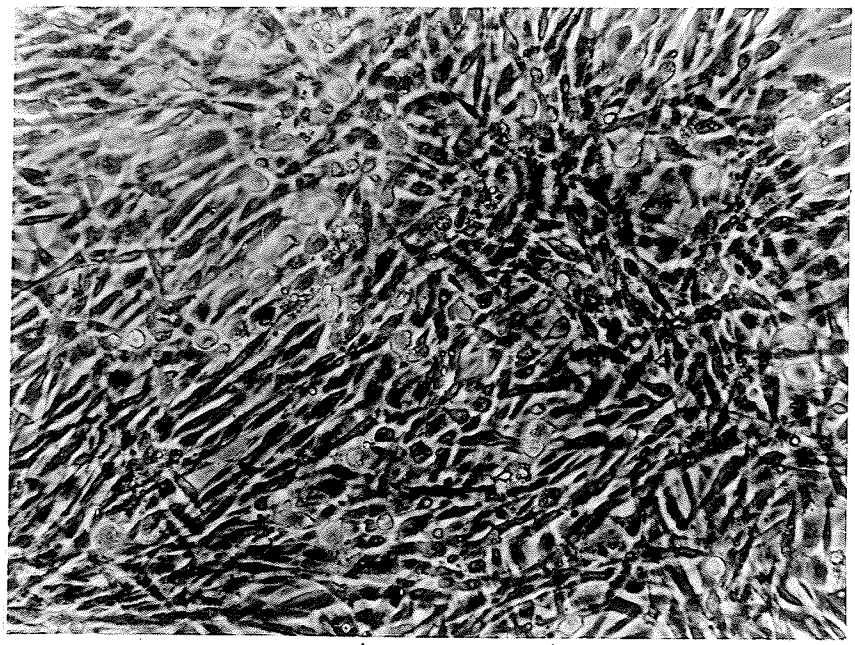
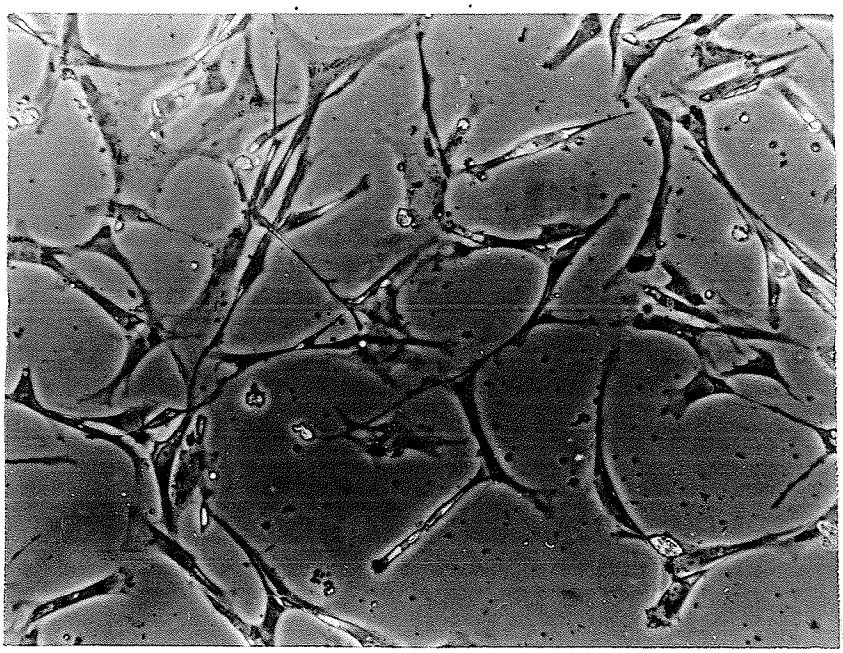


Figure T. Normal control chicken fibroblasts in cell culture on day 2. Cells grown in medium SC6 with 5% chicken serum, 5 ng/ml PMA. Some of the normal control cells have adopted a narrow, spindle shape similar to RSV-infected cells. In general the cells have a narrower shape compared to their "normal" counterparts in moderate density cultures. Phase contrast X100.



TABLES

TABLE 1

MEDIUM CHANGE RESPONSE INDUCED BY DIFFERENT SERA

Test Reagent[a]	Medium Change Response
Human serum	4
5% Human serum[b]	4
Human umbilical cord serum	4
5% Human umbilical cord serum[b]	4
Calf serum	4
5% calf serum[b]	4
Fetal calf serum	4
5% Fetal calf serum[b]	4
Chicken serum	4
5% Chicken serum (Commercial)[b]	4
Chicken serum (fresh)	4
5% Heat-inactivated (fresh) chicken serum[b]	4
Medium SC6 without serum	0

a. 2 ml. of reagent was added to 3-day-old cultures of RSV-infected fibroblasts grown in medium SC6 with 5% chicken serum.

b. Dilutions of sera were prepared in medium SC6.

TABLE 2

MEDIUM CHANGE RESPONSE INDUCED BY OTHER AGENTS

Test reagent[a]	Medium Change Response
5% Heat-inactivated chicken plasma	4
5% $Al(OH)_3$ absorbed heat-inactivated plasma	4
5% chicken serum	4
5% serum of clarified chicken plasma	4
5% deplasminogenated chicken serum	4
SC6 medium	0
1% chicken plasminogen (25% serum:plasminogen)	0
2.5units[b] human plasminogen	0
Bovine serum albumin 2g/litre	0
Human serum albumin 2g/litre	0
Dbc 3', 5' cyclic AMP 10^{-3}M	0

a. 2 ml of reagent was added to 3-day-old cultures of RSV-infected fibroblasts grown in medium SC6 with 5% chicken serum. Dilutions of test reagents were made in medium SC6.

b. (SIGMA) units described in methods and materials.

TABLE 3

EFFECT OF PRE-INCUBATION OF TEST SERUM ON THE MEDIUM CHANGE RESPONSE

Period of incubation[a]	Medium change response
Media incubated on confluent RSV-infected fibroblasts	
10 min	4
30 min	4
1 hr	4
2 hr	4
3 hr	4
4 hr	1
5 hr	0
12 hr	0
24 hr	0
Media incubated on confluent uninfected fibroblasts	
10 min	4
30 min	4
1 hr	4
2 hr	4
3 hr	4
4 hr	1
5 hr	0
12 hr	0
24 hr	0
Control: Medium incubated in cell-free dishes for 24 hours	4

a. Culture dishes kept in warm room

b. Medium SC6 containing 5% chicken serum was incubated for the periods indicated on confluent RSV-infected or uninfected fibroblasts.

TABLE 4

INDUCTION OF MEDIUM CHANGE RESPONSE BY DIALYSATE AND ULTRAFILTRATE

Test reagent[a]	Medium change response
Ultrafiltratrates of chicken serum[b]	
20,000 Dalton cut off	0
20,000 Dalton cut off, 5%	0
50,000 Dalton cut off	0
50,000 Dalton cut off, 5%	0
100,000 Dalton cut off	4
100,000 Dalton cut off, 5%	2
Dialysates of chicken serum[c]	
10,000 Dalton dialysate	4
10,000 Dalton dialysate, 5%	4
Ultrafiltrates of human serum[d]	
50,000 Dalton cut off	0
50,000 Dalton cut off, 5%	0
100,000 Dalton cut off	4
100,000 Dalton cut off, 5%	2

a. 2 ml of reagent was added to 3-day-old cultures of RSV-infected fibroblasts grown in medium SC6 with 5% chicken serum. Dilutions of test reagents were made in medium SC6.

b. Ultrafiltrates of commercial chicken serum with indicated cut off weight.

c. Dialysate of commercial chicken serum at indicated weight.

d. Ultrafiltrate of commercial human serum with indicated cut off weight.

TABLE 5

EFFECT OF HEATING ON ABILITY OF SERA TO INDUCE MEDIUM CHANGE RESPONSE

Test reagent[a]	Medium Change Response
Commercial chicken serum, boiled 1 hour	4
5% Commercial chicken serum, boiled 1 hour[b]	2
Commercial chicken serum, boiled 2 hours	4
5% Commercial chicken serum, boiled 2 hours[b]	2
Commercial human serum, boiled 2 hours	4
5% Commercial human serum, boiled 2 hours[b]	4

a. 2 ml of reagent was added to 3-day-old cultures of RSV-infected fibroblasts grown in medium SC6 with 5% chicken serum.

b. Commercial sera (boiled) diluted in medium SC6.

TABLE 6

MEDIUM CHANGE RESPONSE OF RSV-INFECTED FIBROBLASTS GROWN IN PRESENCE OF A PROTEASE INHIBITOR

	DURATION OF CULTURES							
	DAY 0		DAY 2		DAY 3		DAY 4	
Test reagent[a]	Before	After	Before	After	Before	After	Before	After
Control: Medium SC6 with 5% chicken serum	Flat	4	Flat	4	Few Clumps	4	Many Clumps	4
Protease inhibitor[b]								
Heparin 4 u/ml	Flat	4	Flat	4	Flat	0	Flat	0
Heparin 2 u/ml	Flat	4	Flat	4	Flat	0	Flat	0
Heparin 0.2 u/ml	Flat	4	Flat	4	Few clumps	4	Few clumps	4
EACA 100 mM	Flat	4	Flat	2	Flat little growth	1	Flat reduced growth	1
EACA 10 mM	Flat	4	Flat	1	Flat	0	Flat	1
EACA 1 mM	Flat	4	Flat	1½	Flat	2	Clumps	3
GABA 100 mM	Flat	4	Flat	2½	Flat little growth	0	Flat reduced growth	1
GABA 10 mM	Flat	4	Flat	3	Flat	0	Flat	1½
Trasylol 1000 KIU/ml	Some cells rounded	4	Flat	1	Flat	1	Few clumps	1½
Soybean Trypsin Inhibitor 100 ug/ml	Flat	4	Flat	1½	Flat	1	Flat	2
Lima bean Trypsin Inhibitor 100 ug/ml	Flat	4	Little growth flat	4	Clumps	4	Clumps	4

TABLE 6 (Cont'd.)

	DURATION OF CULTURES							
	DAY 0		DAY 2		DAY 3		DAY 4	
Test reagent[a]	Before	After	Before	After	Before	After	Before	After
Protease inhibitor[b]								
Benzamidine 10^{-4}M	Flat	4	Flat	4	Clumps	4	Cells clumped off	
Leupeptin 10^{-4}M	Flat	4	Flat	½	Flat reduced growth	0	Flat reduced growth	0
Leupeptin 10^{-5}M	Flat	4	Flat	½	Flat	0	Flat	0
Leupeptin 10^{-6}M	Flat	4	Few clumps	1½	Clumps	1	Clumps	2
Antipain 10^{-4}M	Flat	4	Flat little growth	1	Flat Reduced growth	0	Flat Reduced growth	0
Antipain 10^{-5}M	Flat	4	Flat	1½	Flat	1	Flat	2
Antipain 10^{-6}M	Flat	4	Clumps	2	Clumps	2	Clumps	2½

a. 2 ml of medium containing test reagent added to cultures after removal of old medium on days indicated.

b. Protease inhibitors diluted into medium SC6 with 5% chicken serum.

TABLE 7

MEDIUM CHANGE RESPONSE OF RSV-INFECTED
FIBROBLASTS GROWN IN PRESENCE OF A PROTEASE INHIBITOR(S)

Test reagent[a]	DURATION OF CULTURES							
	DAY 0		DAY 2		DAY 3		DAY 4	
	Before	After	Before	After	Before	After	Before	After
Control: Medium with SC6 with 5% heat-inactivated chicken plasma	Flat	4	Flat	4	Clumps	4	Many Clumps	4
Protease inhibitor[b]								
Heparin 2u/ml	Flat	4	Flat	½	Flat	0	Flat	0
Lysine 10 mM	Flat	4	Flat	1	Flat	2	Clumps	3
EACA 100 mM	Flat	4	Flat	2	Flat	½	Reduced growth	0
EACA 10 mM	Flat	4	Flat	1	Flat	1	Flat	0
EACA 1 mM	Flat	4	Flat	2	Clumps	4	Clumps	4
EACA 10mM + Heparin 2 units/ml	Flat	4	Flat	2	Flat	1	Flat	2
EACA 10 mM + Trasylol 1000 KIU/ml	Flat	4	Flat	3	Few Clumps	1	Few Clumps	1
Trasylol 1000 KIU/ml	Flat	4	Flat	3	Few clumps	1	Few clumps	1
Leupeptin 10^{-5}M	Flat	4	Flat	1½	Flat	0	Flat	0

TABLE 7 (Cont'd.)

Test reagent[a]	DURATION OF CULTURES							
	DAY 0		DAY 2		DAY 3		DAY 4	
	Before	After	Before	After	Before	After	Before	After
Medium[c]								
Medium SC6, calcium-free with 5% heat-inactivated chicken plasma	Flat	2	Flat	1	Flat	1	Flat	½
Medium SC6 with 5% Heparinized (40u/ml) chicken plasma	Flat	4	Flat	½	Flat	0	Flat	0
Medium SC6 with 5% Heparinized (102 u/ml) chicken plasma	Flat	4	Flat	½	Flat	0	Flat	0
Medium SC6 with 5% Amphojel absorbed heat-inactivated chicken plasma	Flat	4	Flat	4	Clumps	4	Clumps	4

a. 2 ml of medium containing test reagent added to cultures after removal of old medium on days indicated.

b. Protease inhibitors diluted into medium SC6 with 5% heat-inactivated chicken plasma.

c. Complete medium, test reagent inclusive.

TABLE 8

MEDIUM CHANGE RESPONSE OF RSV-INFECTED FIBROBLASTS ON ADDITION OF PROTEASE INHIBITORS

Test reagent[a]	Medium change response
Control: Medium SC6 with 5% commercial chicken serum	4
Medium SC6 with 5% heparinized (40 units/ml) plasma	4
Protease inhibitor[b]	
Heparin 1000 units/ml	4
Heparin 2 units/ml	4
Heparin 2 units/ml[c]	4
EACA 100 mM	4
EACA 10 mM	4
EACA 10 mM[c]	4
Leupeptin 10^{-3}M	4
Leupeptin 10^{-4}M	4
Leupeptin 10^{-4}M[c]	4
TLCK 1 mg/ml	4
TLCK 100ug/ml	4

a. 2 ml of reagent added to 3-day-old cultures of RSV-infected fibroblasts grown in medium SC6 with 5% chicken serum.

b. Protease inhibitor diluted in medium SC6 with 5% chicken serum.

c. Prepared 5 days before use, others prepared on day of use.

MEDIUM CHANGE RESPONSE OF RSV-INFECTED FIBROBLASTS
GROWN IN PRESENCE OF REDUCED CALCIUM

Medium[a]	DURATION OF CULTURE							
	DAY 0		DAY 2		DAY 3		DAY 4	
	Before	After	Before	After	Before	After	Before	After
Medium SC6 with 5% chicken serum	Flat	4	Flat	4	Clumps	4	Many Clumps	4
Medium SC6, calcium-free with 5% chicken serum	Flat	2½	Flat	½	Flat	1	Flat	1
Medium SC6, calcium-free with 5% 2.5 mM EGTA and with 5% chicken serum	Flat	4	Flat	½	Few round cell reduced growth	1	Rounded cells reduced growth	3
Medium SC6, calcium-free, reduced magnesium with 5% chicken serum[b]	Flat	2½	Flat	½	Flat	1	Flat	1
Medium SC6, calcium-free with 5% chicken serum +10mM EACA[c]	Flat	4	Flat	½	Flat	½	Flat	0
Medium SC6-calcium-free with 5% chicken serum + 2units/ml Heparin[c]	Flat	4	Flat	1	Flat	0	Flat	0

a. 2 ml of medium added to cultures after removal of old medium on days indicated. All cultures seeded in medium SC6, calcium free with 5% chicken serum.

b. Reduced magnesium concentration in this medium is mg^{2+} = 0.15 mM.

c. Protease inhibitor diluted into medium SC6, calcium free with chicken serum.

TABLE 10

MEDIUM CHANGE RESPONSE OF RSV-INFECTED FIBROBLASTS ON ADDITION OF CULTURE MEDIUM CONTAINING REDUCED CALCIUM CONCENTRATION, CALCIUM ANTAGONIST OR CALCIUM IONOPHORE

Medium[a]	Medium Change Response
Medium SC6 with 5% heat-inactivated chicken plasma	4
Medium SC6, calcium-free with 5% heat-inactivated chicken plasma	1½
Medium SC6 with heat-inactivated chicken plasma +D-600 10^{-4} M[b]	4
Medium SC6 with 5% chicken serum	4
Medium SC6, calcium-free with 5% chicken serum	1½
Medium SC6, calcium-free, reduced magnesium with 5% chicken serum[c]	1½
Medium SC6, calcium free with 5% 2.5 mM EGTA and with 5% chicken serum	½
Medium SC6, calcium-free with 10% 2.5 mM EGTA and with 5% chicken serum	2½
Medium SC6, calcium-free with 5% 2.5 mM EGTA with 5% chicken serum + 2 units/ml heparin	½
Medium SC6	0
Medium SC6, calcium free	0
Medium SC6, Divalent Ionophore A23187, 10^{-5} M[d]	0
Medium SC6, Divalent Ionophore A23187, 10^{-6} M[d]	0

a. 2 ml of medium added to 3-day-old cultures of RSV-infected fibroblasts grown in medium SC6 with 5% chicken serum. Cultures washed twice with PEG-CF before test medium added.

b. D-600 a calcium antagonist diluted into medium.

c. Reduced magnesium concentration in medium is $\left[Mg^{2+}\right]$ = 0.15 mM

d. Divalent ionophore diluted in medium SC6.

TABLE 11

MEDIUM CHANGE RESPONSE OF RSV-INFECTED FIBROBLASTS IN PRESENCE OF OTHER REAGENTS

	DURATION OF CULTURES							
	DAY 0		DAY 2		DAY 3		DAY 4	
	Before	After	Before	After	Before	After	Before	After
Control: Medium SC6 with 5% chicken serum	Flat	4	Flat	4	Clumps	4	Many Clumps	4
Dbc 3', 5'-cyclic AMP $10^{-4}M$[b]	Flat	4	Flat Some Rounded	4	Clumps	4	Floating Cells Many clumps	4
Dbc 3', 5'-cyclic AMP $10^{-5}M$[b]	Flat	4	Flat Some Rounded	4	Clumps	4	Floating Cells Many clumps	4
5'-AMP $10^{-5}M$[b]	Flat	4	Flat Some Rounded	4	Clumps	4	Many clumps	4

a. 2 ml of medium containing test reagent added to cultures after removal of old medium on days indicated.

b. Test reagent diluted in medium SC6 with 5% chicken serum.

TABLE 12

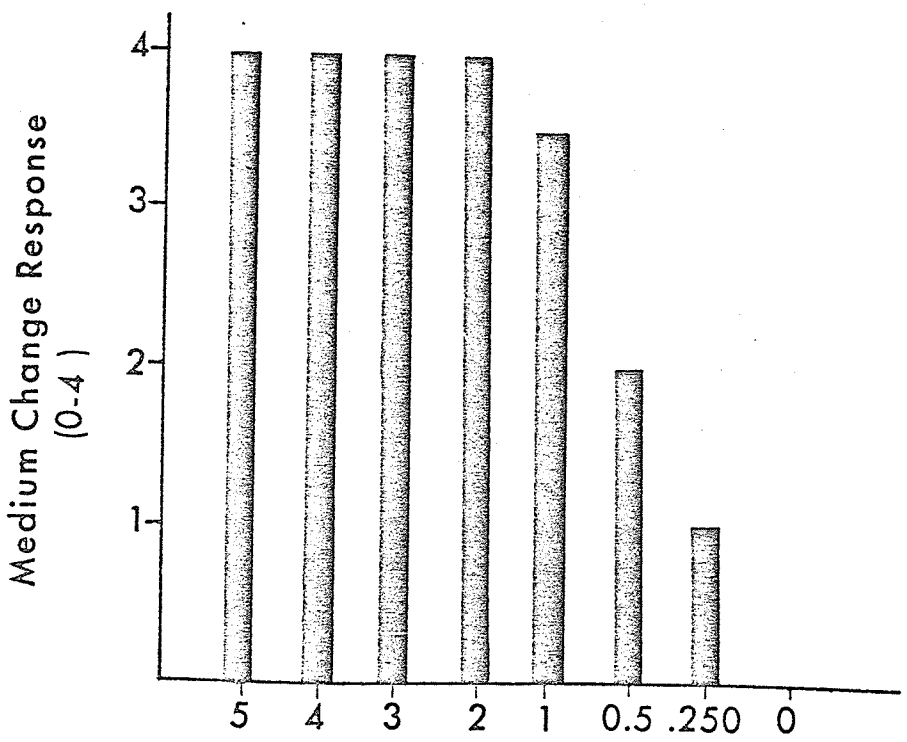
EFFECT OF REPEATED CULTURE MEDIUM CHANGES ON RSV-INFECTED FIBROBLASTS

Observation time (hours)[a]		1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.
1	Before	Partially rounded cells					
	After	0					
2	Before	Flat, not fully elongated	Flat				
	After	0	0				
3	Before	Flat		Flat			
	After	0		0			
4	Before	Flat	Flat		Flat		
	After	0	1		1		
5	Before	Flat				Flat	
	After	½				4	
6	Before	Flat	Flat	Flat			Flat
	After	½	½	2			4

a. 2 ml of medium SC6 with 5% chicken serum added to 3-day-old RSV-infected fibroblasts after removal of old medium. Initial change at "0" hour produces MCR of 4. Repeated culture medium changes are made then at indicated time intervals. Cultures are observed before and after medium change. After each medium change MCR indicated.

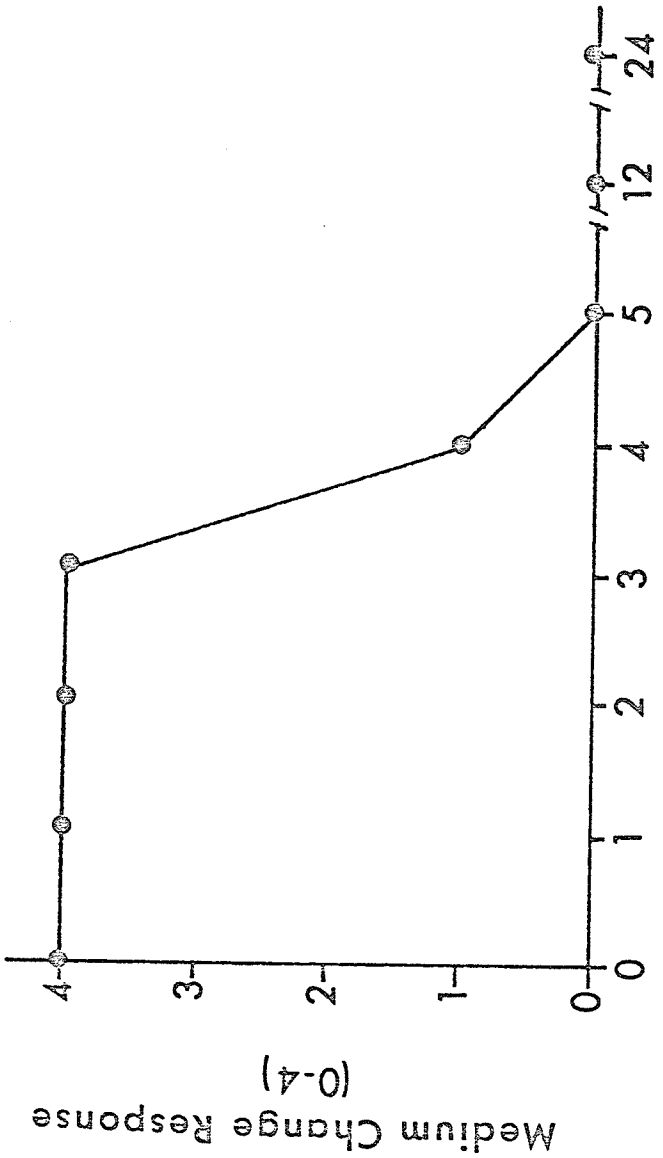
Graph 1. The MCR of RSV-infected chicken fibroblasts on addition of 2 ml. of synthetic medium (SC6) containing chicken serum at concentrations of 5% to 0.25%.

Spent culture medium was aspirated off the culture dish before fresh culture medium was added. Each bar represents two separate MCR occurring in two culture dishes containing RSV-infected fibroblasts upon the addition of the particular chicken serum concentrations in medium SC6.


Medium Change Response
(0-4)
4
3
2
1
5
4
3
2
1
0.5
.250
0
Medium: % of serum in SC6
2mls added per dish

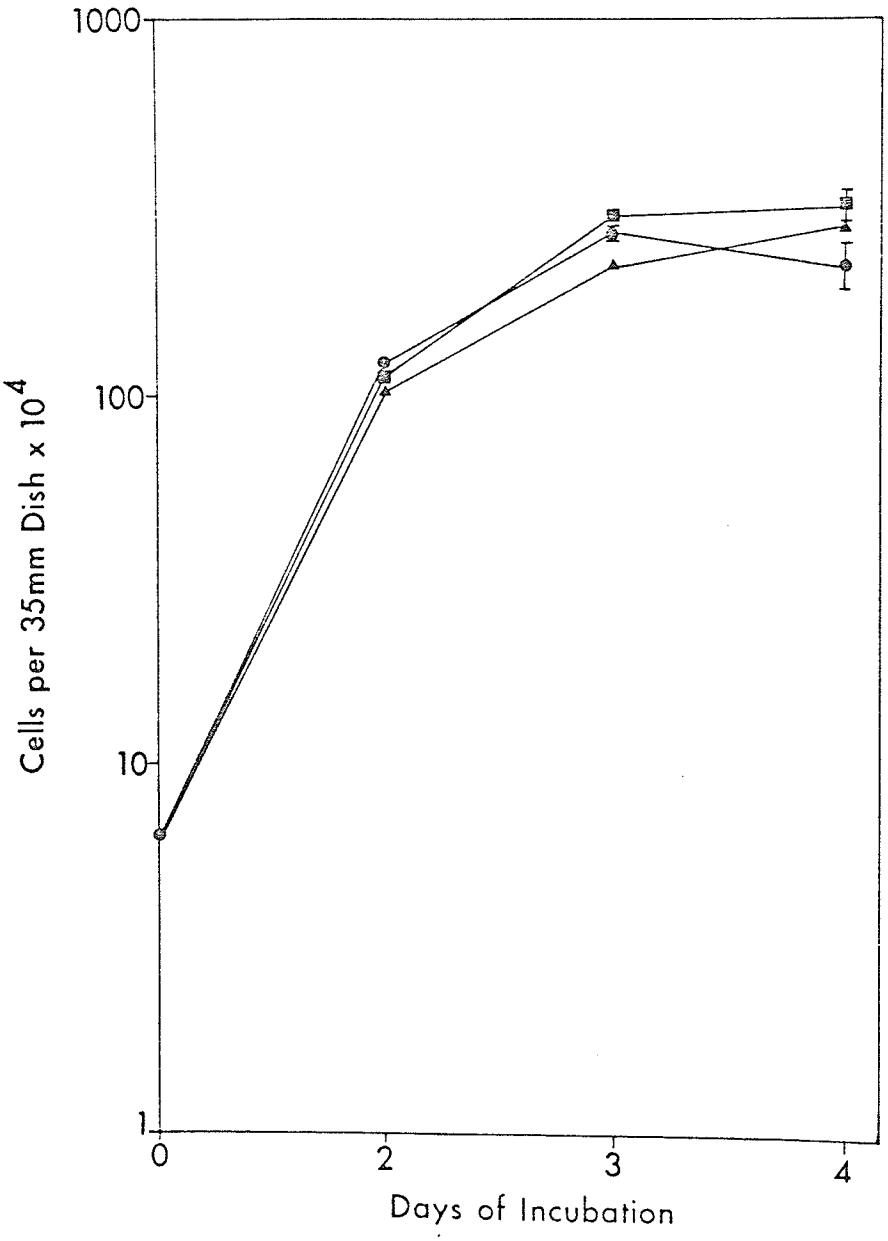
Graph 2. MCR of RSV-infected chicken fibroblasts on addition of cell (RSV-infected fibroblasts) incubated culture medium.

Fresh medium SC6 with 5% chicken serum was added to culture dishes containing high or moderate density RSV-infected fibroblasts. The culture medium is left on these dishes for a time period (culture dishes kept in the warm room during this time period) and the medium was aspirated off. This aspirated culture medium was collected and added to 3-day-old RSV-infected fibroblast cultures as a medium change. This graph corresponds to Table 3.


Medium Change Response
(0-4)
0
1
2
3
4
0
1
2
3
4
5
12
24
Time (hours)
Period of culture medium incubation on RSV-infected
fibroblasts before transfer

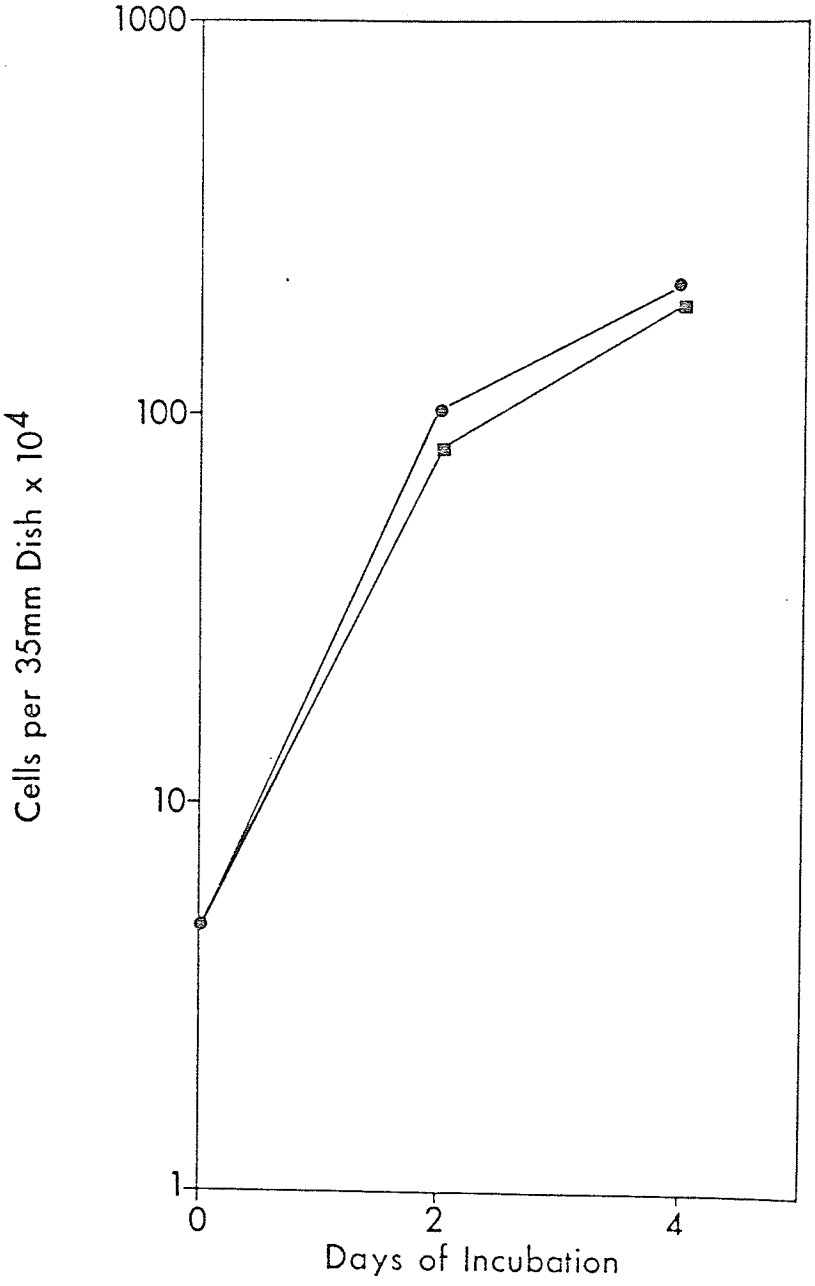
Graph 3. Effect of leupeptin and heparin in medium SC6 with 5% chicken serum on the proliferation of RSV-infected chicken fibroblasts.

On the day following passage (day 0), cultures were changed to (●) control medium (SC6 with 5% chicken serum), (■) control medium with 10^{-5}M leupeptin or (▲) control medium with 2 units/ml heparin. Culture media were changed on days 2 and 3. Each point represents the mean ± SEM of 2 culture dishes.

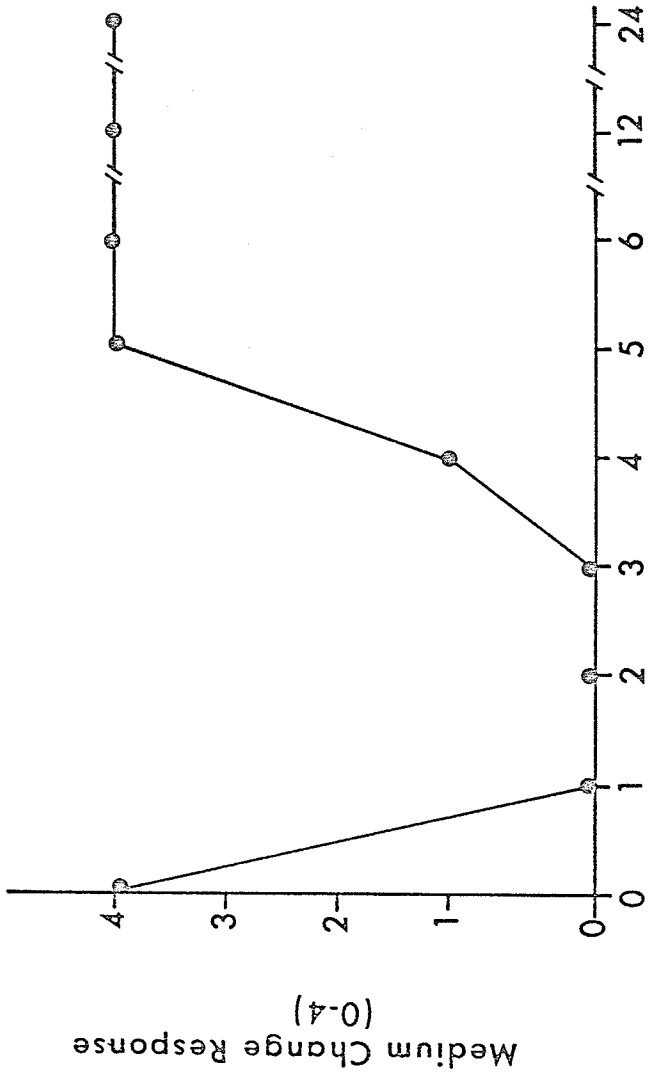

1000
100
10
1
Cells per 35mm Dish x 10^4
0
2
3
4
Days of Incubation

Graph 4. Effect of leupeptin in medium SC6 with 5% heat-inactivated chicken plasma on the proliferation of RSV-infected chicken fibroblasts.

On the day following passage (day 0) cultures were changed to (●) control medium (SC6 with 5% heat-inactivated chicken plasma), (■) control medium with 10^{-5}M leupeptin. Culture media were changed on days 2 and 3. Each point represents the mean ± SEM of 2 culture dishes.


1000
100
10
1
0
2
4
Days of Incubation
Cells per 35mm Dish x 10^4

Graph 5. MCR of RSV-infected chicken fibroblasts on addition of medium SC6 with 5% chicken serum after a time interval from a previous culture medium change. On several culture dishes culture medium (SC6 with 5% chicken serum) is changed at 0 hours. After a 1 hour time interval two culture dishes' medium were changed to fresh medium SC6 with 5% chicken serum and the MCR was observed. This procedure is repeated at the 2-hour time interval on dishes that have not been changed since 0 hour. The procedure is repeated up to a 24-hour time interval. This graph corresponds to Table 12.



Medium change at time intervals on separate dishes after initial medium change at 0 hour

DISCUSSION

DISCUSSION

Rous sarcoma virus-infected fibroblasts differ in characteristics from their normal counterpart fibroblasts; one of these characteristics is clumping and rounding of RSV-infected cells while normal counterparts remain flat in moderate or high cell densities. Through coincidence, RSV-infected cells were observed immediately after a culture medium change and were found to undergo a rapid rounding and aggregation. This unique observation of RSV-infected fibroblasts has been called the "medium change response" (MCR). The phenomenon has not been previously reported. I have attempted to characterize and find the cause of this phenomenon.

Cell-Shape Changes Due to the MCR

The rapid rounding of RSV-infected fibroblasts after culture medium change is very similar to what is seen when trypsin is added to fibroblasts grown in a cell culture dish. Trypsinized fibroblasts first undergo blebbing of the plasma membrane and then acquire a round shape. This sequence of events has also been observed in the MCR. The rounded form is thought by some to be a consequence of surface tension (112). This has, however, been shown not to be the cause of fibroblasts rounding after trypsinization. There is evidence that there is no change of surface area in the transition from round to flattened cells (113). Trypsinized cells viewed under a scanning electron microscope show a highly convoluted and blebbed surface

(112). Most likely the rounding of cells during trypsinization is due to subsurface tensions produced by contraction of cortical actomyosin upon exposure to trypsin.

The MCR does not occur when spent plasma or serum containing medium from RSV-infected cells incubated for 6 hours is aspirated from and then returned to cultures or when synthetic medium with albumin is added. These findings indicate that the MCR does not have a physical basis. Also the negative response on addition of albumin indicates that the phenomenon does not represent a non-specific reaction to the addition of fresh protein. An interesting observation that may be related to RSV-infected fibroblast cell behaviour is that after cells have undergone a MCR, some remain rounded-up forming aggregates. On the next day these aggregates of rounded cells are still present, but with elongated spindle-shaped cells on the bottom of the aggregates, projecting like rays and giving a stellate appearance. An explanation may be that the rounded cells in the aggregates cannot flatten out on each other but require a suitable surface for attachment before the flattening process will occur.

Plasma and Serum Induce the MCR

Chicken serum and plasma, along with bovine and human serum, were found to induce the MCR. This information indicates that the inducer is ubiquitous in serum of various species. Culture medium containing chicken serum in a concentration as low as 1% could produce a full strength MCR. This could mean that there is a large quantity of this inducer present or that

a small amount of inducer can trigger a potent chain reaction. Ultrafiltration of chicken or human serum showed that the induction of the MCR involves a serum (plasma) macromolecule(s) in the approximate molecular weight range of 50,000-100,000 daltons. The inducer macromolecule(s) seems to have considerable heat stability. These physical characteristics of the inducer macromolecule(s) narrow the range of possible candidate macromolecule(s) that could cause the MCR.

Through past literature review and MCR results plasminogen was considered as a prime candidate. M. Weber has reported that the appearance of "transformation" specific changes in morphology and adhesiveness of RSV-T5 (temperature sensitive virus mutant)-infected chick embryo fibroblasts is dependent on the presence of plasminogen (100). In my results deplasminogenated chicken serum gave a strong MCR while the addition of chicken and human plasminogen in synthetic medium gave no response. These results essentially exclude the possibility that plasminogen or plasmin are involved in the MCR. Plasminogen activator from RSV-infected chick fibroblasts can catalytically convert plasminogen rapidly into plasmin (72).

J. Quigley has shown that adding plasmin directly to RSV-infected chick embryo fibroblasts will transiently induce cell clustering of flat monolayer cultures. He reports also that in the plasmin-treated cultures, clustering did not persist and they detached from the culture dish after 2-4 hours. As these results are contrary to mine, it is possible that RSV-chick embryo fibroblasts respond to plasmin differently than to RSV-chicken fibroblasts and are producing a different version

of the MCR phenomenon.

Effects of Protease Inhibitors and Calcium on the MCR

Leupeptin 10^{-5}M and heparin 2 units/ml were found to be the best protease inhibitors that would abolish the MCR in a 4-day cultivation period. Higher concentrations of leupeptin or heparin would slow down the growth rates of the cells and would not abolish the MCR any quicker. At these high or low concentrations no altered morphology of the RSV-infected fibroblasts was seen which is an indication that the increased adherence of the RSV-infected cells is not due to conversion into "normal" cells.

There were other protease inhibitors such as EACA, trasylol and soybean trypsin inhibitor that would only partially inhibit the MCR over a 4-day cultivation period. The abolition or diminution of the MCR by these various protease inhibitors used in these experiments suggests that a serine protease is involved in the generation of the MCR. The observation that the MCR is abolished after RSV-infected fibroblasts have been cultivated for a period of 3 days in the presence of leupeptin or heparin also suggests there is a cellular serine protease(s) involved in activation of the MCR.

Protease inhibitors at high concentrations added to the cultures for the first time with fresh serum or heat-inactivated plasma-containing medium were unable to abolish the MCR. This finding indicates that these agents do not act by binding to any serum (plasma) component but, rather, that they act on the

cultured RSV-infected fibroblasts themselves. Inhibition of the MCR by protease inhibitors seems to be a slow process; at least two days are required before a reduction of the MCR occurs. RSV-infected cells cultivated in protease inhibitors for 1 day show no reduction in MCR.

The use of a wide range of serine protease inhibitors with certain specificities has given some hint as to the type of serine protease probably involved in the MCR. Leupeptin, a plasmin and trypsin inhibitor, was one of the best MCR inhibitors used. This inhibitor is an arginine-containing peptide aldehyde. Antipain, another peptide aldehyde, reduced the MCR partially. Benzamidine, an arginine analogue, however, did not reduce the MCR. Trasylol and soybean trypsin inhibitor are general inhibitors of trypsin-like enzymes, only partially inhibited the MCR. EACA, a lysine analogue, is a plasmin and plasminogen inhibitor that caused a good reduction of the MCR. Lysine caused less reduction. GABA which has a similar structure to EACA but with a shorter carbon chain, results in a partial inhibition. Most of these serine protease inhibitors are small molecules and can enter the cell quite easily. The results from the use of the above serine protease inhibitors indicate that the possible serine enzyme involved has a plasmin or trypsin type of activity.

Another serine protease inhibitor quite different than the above, and that also abolishes the MCR, is heparin, a thrombin enzyme inhibitor. Heparin is a unique serine protease inhibitor in that it has a large anion charge, the ability to

bind to and inhibit the activity of a wide variety of functional proteins (110). Due to the size of the heparin molecule one could make an assumption that this serine protease inhibitor reacts with the cellular protease(s) involved in the MCR on the membrane surface rather than entering the cell and acting intracellularly like the other serine protease inhibitors. Alternatively, heparin may inhibit a critical step in the MCR by binding to the surface or other structural components of RSV-infected fibroblasts.

Increased blebbing has been observed when culture medium is changed on cells cultivated with protease inhibitors such as EACA and heparin and in low calcium culture medium. This blebbing phenomenon is possibly more prominent because these protease inhibitors or low calcium culture media are preventing the process of cell rounding at a stage just after blebbing. Blebbing seems to be a result of the cell's plasma membrane and cortical cytoplasm involuting due to disruption of the cortical cytoskeleton structures. In cultures grown in complete culture medium without protease inhibitors, the addition of serum or plasma medium causes the cells to undergo a rapid rounding thus allowing little time to observe blebbing.

Leupeptin 10^{-5}M and heparin 2 units/ml could abolish the MCR after 3 days cultivation in serum or plasma-containing medium without affecting the proliferative rate of the cells. Cultivation of cells with protease inhibitors at these concentrations does not affect growth rates, thus indicating that there is no toxicity. From these observations one can say that the MCR may not be involved in the maintenance of autonomous pro-

liferation of RSV-infected fibroblasts.

The marked reduction in strength of the MCR that is observed with the addition of serum-containing medium of reduced calcium concentration, as compared to medium of physiological calcium concentration, suggests that the MCR involves this ion.

Calcium ion may be a co-factor (for the serine protease that appears to be) involved in protease release or in activation of the contractile system of RSV-infected fibroblasts. The failure of ionophore A23187 to induce the MCR implicates the former set of possibilities more than the latter, i.e. the lack of activity of the ionophore suggests that calcium may be involved in the evolution, rather than in the initiation, of the MCR. This result may indicate that the MCR does not involve increase of cytoplasmic calcium ion.

D-600 is an antagonist of dynamic, depolarization-activated Ca^{2+} influx in cell membranes (117). When D-600 was added to cells there was no reduction of the MCR. This result may indicate that the MCR does not depend on calcium influx. From the results of ionophore and D-600 one can assume the MCR mechanism does not depend on the enterance of calcium ion into the cell.

Effect of Phorbol-12-myristate-13-acetate Inducing a MCR

Sarcoma virus-infected fibroblasts synthesize and release a variety of serine proteases; well represented among these are plasminogen activator(s) (69). Tumor promoter phorbol-12-myristate-

13-acetate (PMA) has been shown to be an inducer of tumors in a two-stage carcinogenesis system (114). Increased plasminogen activator(s) levels (10 fold) have been reported in chick embryo fibroblast cultures that have been treated with PMA (115). In my experiments morphological changes (spindle-shaped cells) were observed upon the addition of 5-50 ng/ml PMA to "normal" chicken fibroblasts. These findings were similar to that J. Quigley reported when adding PMA to chick embryo fibroblasts (116). In both studies no clustering or aggregation of cells was observed. Cultivation of "normal" cells with PMA in my study did not produce the MCR. J. Quigley's, Schmidt-Ruppin Rous sarcoma virus-infected chick embryo fibroblasts do not show cell clustering or aggregation until the addition of PMA, however, he does not report a MCR phenomenon. In his culture system the RSV-infected chick embryo fibroblasts were treated with PMA in serum-free culture medium for several days. The PMA effect on RSV-chick embryo fibroblasts could be inhibited by leupeptin (10^{-3}M), soybean trypsin inhibitors (100 u/ml) and benzamidine (10^{-3}M). No effective inhibition of cell clustering or aggregation occurred upon addition of Trasylol (100 u/ml), antipain (10^{-4}M) and EACA (10^{-2}M) (116). These protease inhibitor effects were observed after 24 hours (116).

In J. Quigley's PMA cell cultivation one can question the use of growing cells in serum-free culture medium. In the RSV-infected chicken fibroblast system, if the cells were grown in serum-free medium for a couple of days they would round up and detach due to the affect of starvation. Plasminogen activator has been associated with the morphologic changes that

occur in the RSV-infected chick embryo fibroblasts upon PMA addition (114, 116). Plasminogen activator may possibly be involved in the MCR; an assay system is currently being developed. There is a possibility that J. Quigley's RSV-infected chick embryo fibroblasts have a lower level of plasminogen activator(s) than the RSV-infected chicken fibroblasts. In his system the addition of PMA to his RSV-infected cells may increase the level of plasminogen activator(s) to the level present in the RSV-infected chicken fibroblasts. A certain level of plasminogen activator may be needed before cell clustering will occur. An alternate possibility is the increase of the production of another serine protease that is also present in large quantities in RSV-infected fibroblasts.

The effect of serine protease inhibitors such as leupeptin and soybean trypsin inhibitor in his system were similar to the results in my system. These results give further evidence that plasminogen activator is involved in the morphological changes, however, EACA and trasylol were not effective in his system. There is a possibility of another serine protease involved, that has similar characteristics to plasminogen activator.

The MCR in a In Vivo Situation

In the in vitro system I have studied, the MCR is induced by the replacement of spent culture medium with fresh culture medium. The active principal appears to be a serum (plasma) macromolecule(s). In vivo, however, sarcomatous fibroblasts, like normal fibroblasts are exposed to a continuous supply of fresh plasma proteins. (Plasma proteins are present at least

10% of their plasma concentrations in the normal interstitial fluids; ref. 14).

I have attempted to mimic the *in vivo* situation by changing cultures of RSV-infected fibroblasts to fresh medium at short intervals after they have sustained an initial MCR. The results suggest that the MCR, as observed *in vitro*, may represent an abrupt and highly accentuated form of a phenomenon that occurs at a continuous low level *in vivo*. Possibly, under the influence of a plasma macromolecule sarcoma cells *in vivo* continuously release small quantities of a protease into their environment; such a protease may be abruptly released *in vitro*, when culture medium that has become depleted of the plasma inducer is replaced with fresh medium that contains the inducer.

Another characteristic of the MCR is that after 5 hours of exposure to moderate density cultures of RSV-infected or "normal" fibroblasts, serum (plasma) containing culture medium will no longer induce the MCR when transferred to other dishes of RSV-infected fibroblasts. This observation suggests that the serum component(s) that induces the MCR is consumed or inactivated, by both cell types.

Three Possible Mechanisms and Significance of the MCR

One of at least three basic mechanisms may be operative in the generation of the MCR: (a) Addition of the serum (plasma) inducer may lead to abrupt liberation of a serine protease from RSV-infected fibroblasts into their immediate environment. Such a protease might then cleave the attachments of these cells to the culture surface, followed by cellular rounding and aggre-

gation; (b) the serum (plasma) inducer might be a proenzyme (other than plasminogen) that is activated by a serine protease residing in the surface of the RSV-infected fibroblasts. The activated proenzyme might then cause cellular detachment from the culture surfaces; (c) addition of the serum inducer might activate contractile elements within the RSV-infected fibroblasts, with subsequent cellular rounding, detachment and aggregation. This mechanism would have to involve a serine protease, at some point, because of serine protease inhibitor effects of abolishing the MCR. Also there is a possibility the serine protease inhibitors can interfere with a structural component of the cytoskeletal contractile system. There is a compound W-7((6-aminohexyl)-5-chloro-1-napthalenesulfonimide) which has an EACA like structure. This compound can inhibit actin and myosin interaction in smooth muscle (118). Further study has to be done to see if EACA by itself can produce similar results on smooth muscle contractile system.

The first possible mechanism of the MCR, i.e. protease release in response to a plasma molecule, is conceptually the simplest of the three. Plasminogen activator is known to be released by RSV-infected fibroblasts at higher levels than normal counterparts. Future work will involve isolating the inducer serum (plasma) macromolecule. This will help in identifying the protease being released if there is one.

The significance of the MCR is that this phenomena possibly contributes to the malignant behaviour of sarcomatous fibroblasts. For example <u>in vivo</u> when a RSV-induced tumor in

the fowl reaches a certain developmental stage the tumor cells could produce a MCR upon exposure to the serum (plasma) inducer. Upon producing a MCR the tumor cells could be released from the tumor body and metastasize. Another possibility is that the macromolecule(s) in serum (plasma) is a chemotactin released from a tissue, that induces the rounding of the neoplastic cells thus allowing metastasis. Further work will have to be done to determine what significance the MCR has *in vitro* and *in vivo*.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Braun,C.A., The Story of Cancer, 1st Edition, Addison-Wesley, p. 9, (1977).

2. Rous, P.J., J. Exp. Med., 12, 696 (1910).

3. Carrel C., Burrows M., J. Exp. Med., 13, 571 (1911).

4. Fischer, A., Arch. Entwicklungsmech, 104, 210 (1925).

5. Astrup, T., Permin, P., Nature, 159, 681 (1947).

6. Unkeless, J.C., Tobia, A., Ossowski, L., Quigley, J.P., Rifkin, D.E., Reich, E., J. Exp. Med., 137, 85 (1973).

7. Ossowski, L., Unkeless, J.C., Tobia, A., Quigley, J.P., Rifkin, D.B., Reich, E., J. Exp. Med., 137, 112 (1973).

8. Unkeless, J.C., Dan, K., Kellerman, G.M., Reich, E., J. Biol. Chem., 249, 4295 (1974).

9. Braun, C.A., The Story of Cancer, 1st Edition, Addison-Wesley, p. 172 (1977).

10. Fenner, F., Intervirology 7 (1-2), 53 (1976).

11. Braun, C.A., The Story of Cancer, 1st Edition, Addison-Wesley, p. 173 (1977).

12. Stoker, M., Proc. R.Soc. Lond. B.Sci. 181, 1 (1972).

13. Braun, C., The Story of Cancer, 1st Edition, Addison-Wesley. Chapter VI (1977).

14. Balk, S.D., Role of Calcium, Serum, Plasma, and Folic Acid in the Control of Proliferation of Normal and Rous Sarcoma Virus-Infected Chicken Fibroblasts (Ph.D. Dissertation, Rockerfeller University), (1973).

15. Freeman, A., Huebner, R., J. Nat. Cancer Inst. 50, 303 (1973).

16. Shields, R., Nature, 262, 348 (1976).

17. Stiles, C., Desmond, W., Chuman, L., Sato, G., Saier, M., Cancer Res., 36, 3600 (1976).

18. Stiles, C., Desmond, W., Sato, G., Saier, M., Proc. Nat. Acad. Sci. (U.S.A.), 72, 4971 (1975).

19. Purchase, H.B., Okazaki, W., Voqt, P., Hanafusa, H., Burmester, B. and Crittenden, L., Infect. Immun. 15, 423 (1977).

20. Balk, S., Mitchell, R.S., LeStourgeon, D., Failed Cellular Calcium Homeostasis as the Basis for the Neoplastic State (unpublished).

21. Hanafusa, H., in Comprehensive Virology, Plenum Press, Vol. 10, p. 341 (1977).

22. Temin, H.M., Rubin, H., Virology 6, 669 (1958).

23. Hanafusa, H., Proc. Nat. Acad. Sci. (U.S.A.), 63, 318 (1969).

24. Elsdale, T., Bard, J., Nature, 236, 152 (1972).

25. Hanufusa, H., in Comprehensive Virology, Plenum Press, Vol. 10 p. 448 (1977).

26. Ericksen, S., Eng, J., Morgan, H.R., J. Exp. Med., 114, 435 (1961).

27. Bosmann, H.B., Case, K.R., Morgan, H.R., Exp. Cell Res., 83, 15 (1974).

28. Hakomori, S., Virology, 44, 609 (1971).

29. Hamomori, S., Saito, T., Voqt, P.K., Biochem. Biophys. Acta., 417, 55 (1975).

30. Mora, P.T., Brady, R.O., Bradley, R.M., McFarland, V.W., Proc. Nat. Acad. Sci. (U.S.A.),63, 1290 (1967).

31. Yamada, K.M., Olden, K., Nature, 275, 179 (1978).

32. Olden, K., Yamada, K.M., Cell 11, 957 (1977).

33. Hynes, R.O., Ali, I.V., Destree, A.T., Mautner, V., et al. in Fibroblast Surfaces Protein (Annals of the New York Academy of Sciences, 312), The New York Academy of Science, p. 332 (1978).

34. Weber, J.M., Hale, A.H., Lusasso, L., Cell, 10, 45 (1977).

35. Weber, J.M., Hale, A.H., Roll, E.D., in Protease and Biological Control, Cold Spring Harbour Laboratory, p. 915 (1975).

36. Ali, I.U., Mautner, V.M., Lanza, R., Hynes, P.O., Cell 11, 115 (1977).

37. Rees, D.A., Lloyd, C.W., Thom, D., Nature, 267, 124 (1977).

38. Wang, E., Goldberg, A.R., Proc. Nat. Acad. Sci. (U.S.A.), 73, 4065 (1976).

39. Edelman, B.M., Yahara, I., Proc. Nat. Acad. Sci. (U.S.A.), 73, 2047 (1976).

40. Pollack, R. Rifkin, D., Cell, 6, 495 (1975).

41. Clarke, M., Spudich, J., Ann. Rev. Biochem., 46, 797 (1977).

42. Marx, J., Science, 189, 34 (1975).

43. Kapeller, M., Dolvanski, F., Nature (London) New Biol., 235, 184 (1972).

44. Graf, T., Friis, R.R., Virology 56, 567 (1973).

45. Nicholson, B.L., Nature (London), New Biol., 243, 218 (1973).

46. Nicholson, B.L., Nature (Lond.) New Biology, 233, 244 (1971).

47. Havry, P., Defendi, V., Virology, 41, 22 (1970).

48. Burger, M.M., Proc. Nat. Acad. Sci. (U.S.A.), 62, 994 (1969).

49. Bauer, H., Kurth, R., Rosrschneider, L.R., et al. Cold Spring Harbour Symp. Quant. Biol., 39, 57 (1975).

50. Stoker, M., Rubin, H., Nature, 215, 171 (1967).

51. Rubin, H., Exp. Cell Res., 41, 149 (1966).

52. Bader, J.P., J. Cell Physiol., 70, 301 (1967).

53. Temin, H.M., J. Natl. Cancer Inst., 35, 679 (1965).

54. Jainchill, J.L., Todaro, B.L., Exp. Cell Res., 59, 137 (1970).

55. Balk, S.D., Whitfield, J., Youdale, T., Braun, A.C., Proc. Nat. Acad. Sci. (U.S.A.), 70, 675 (1973).

56. Balk, S.D., Hoon, B.S., Mitchell, R.S., LeStourgeon, D., Proc. Nat. Acad. Sci. (U.S.A.), (In press), (1979).

57. Pardee, A.B., In Vitro, 7, 95 (1971).

58. Warburg, O., Science, 123, 309 (1956).

59. Mitchell, R.S., Elgas, R.S., Balk, S.D., Proc. Nat. Acad. Sci. (U.S.A.), 73, 1265 (1976).

60. Quigley, J.P., Ossowski, L., Reich, E.J. Biol. Chem., 249, 4306 (1974).

61. Rifkin, D.B., Loeb, J.N., Moore, G., Reich, E.J., Exp. Med. 139, 1317 (1974).

62. Ossowski, L., Biegel, D., Reich, E., Cell, 16, 929 (1979).

63. Strickland, S., Reich, E., Sherman, I.M., Cell 9, 231 (1976).

64. Wilson, E.L., Dowdle, E., Int. J. Cancer, 22, 390 (1978).

65. Wigler, M., Weinstein, I.B., Nature, 259, 232 (1976).

66. Reich, E., Wilson, E.L., Cancer Res.,39, 1579 (1979).

67. Diamond, L., O'Brien, T.B., Rovera, B., Life Sci.,23 (1978-79).

68. Wolf, B.A., Goldberg, A.R., Proc. Nat. Acad. Sci. (U.S.A.), 73, 3613 (1976).

69. Dano, K., Oronsky, A., Gjedde, S., in Regulatory Proteolytic Enzymes and their Inhibitors, (FEBS, 47, A6), Pergamon Press, p. 113 (1977).

70. Chen, L.B., Buchanan, J.M., Proc. Nat. Acad. Sci. (U.S.A.), 72, 1132 (1975).

71. Neurath, H., Walsh, K.A., Proc. Nat. Acad. Sci. (U.S.A.), 73, 3825 (1976).

72. Reich, E., Rifkin, D.B., Shaw, E., Proteases and Biological Control, Cold Spring Harbour Laboratory (1975).

73. Hartley, B.S., Annu. Rev. Biochem., 29, 445 (1960).

74. Lehninger, A., Biochemistry, 2nd edition, Worth, p.224 (1975).

75. Davie, E.W., Fryikawa, K., Annu. Rev. Biochem., 44, 799 (1975).

76. Wallen, P., Kok, P., Ranby, M., in Regulatory Proteolytic Enzymes and their Inhibitors, (FEBS, 47, A6), Pergamon Press, p. 127 (1977).

77. Lorand, L. (editor), Methods in Enzymology, Proteolytic Enzymes, Vol. XLV, B, Academic Press Inc. (1976).

78. Kaplan, A.P., Austen, K.F., J. Exp. Med., 136, 1378 (1972).

79. Sturkie, P.D., Avian Physiology, 3rd. edition, Springer-Verlag (1976).

80. Didisheim, P.K., Hattori, K., Lewis, J.H., J. Lab. Clin. Med., 53, 866 (1959).

81. Wartelle, O., Rev. Hematol., 12, 351 (1957).

82. Archer, R., in Physiology and Biochemistry of Domestic Fowl, Vol. 11, Chapter 38, Academic Press Inc. (1971).

83. Beers, W.H., Strickland, S., Reich, E., Cell, 6, 387 (1975).

84. Strickland, S., Beers, H.W., J. Biol. Chem., 251, 5694 (1976).

85. Barrett, A.J., Starky, P.M., Biochem. J., 133, 709 (1973).

86. Ambrus, C.M., Ambrus, J.L., Lassman, B.H., Mink, I.B., in Studies in the Mechanisms of Action of Inhibitors of the Fibrinolysin System (Annals of the New York Academy of Sciences, 146), The New York Academy of Science, p.430 (1968).

87. Kassell, B., Luskoriski, M., J. Biol. Chem., 219, 203 (1956).

88. Shaw, E., Mares-Guia, M., Cohen, W., Biochemistry, 4, 2219 (1965).

89. Roffman, S., Sanocka, H., Troll, W., Anal, Biochem., 36, 11 (1970).

90. Kunitz, M., Northrop, J.H., J. Gen. Physiol., 19, 991 (1936).

91. Mizamoto, M., Lee, K., J. Pharm, Sci., 51, 912 (1962).

92. Christensen, U., Biochimica et Biophysica Acta., 526, 194 (1978).

93. Rosenberg, D., Semin, Hematol.,14, 427 (1977).

94. Damus, P.S., Hicks, M., Rosenberg, R.D., Nature, 246, 355 (1973).

95. Hirsh, J., Brain, A.E., Skov, K.C. (editors), Concepts in Hemostasis and Thrombosis, McMaster University, Hamilton (1976).

96. Sefton, B.M., Rubin, H., Nature, 227, 843 (1970).

97. Schnebli, H.P., Burger, M.M., Proc. Nat. Acad. Sci. (U.S.A.), 71, 1748 (1974).

98. Chou, I.N., Black, P.H., Roblin, R.O., Proc. Nat. Acad. Sci. (U.S.A.), 71, 1748 (1974).

99. Weber, M.J., in Mechanisms of Virus Disease, W.A. Benjamin, p. 327, (1974).

100. Weber, M.J., Cell, 5, 253 (1975).

101. Schnebli, H.P., in Proteases and Biological Control, Cold Spring Harbour Laboratory, p. 785 (1975).

102. Hanafusa, H., Proc. Nat. Acad. Sci. (U.S.A.), 63, 318 (1969).

103. Vogt, P.K., Adv. Cancer Res., 11, 293 (1965).

104. Temin, H., Adv. Cancer Res., 19, 47 (1974).

105. Vogt, P.K., in Comprehensive Virology, Plenum Press, Vol. 9, p. 341 (1977).

106. Collett, M.S., Erickson, E., Erickson, R.L., J. Virol., 29, 770 (1979).

107. Collett, M.S., Erickson, R.L., Proc. Nat. Acad. Sci. (U.S.A.), 75, 2021 (1978).

108. Stehelin, D., Varmus, H.E., Bishop, J.M., Vogt, P.K., Nature, 260, 170 (1976).

109. Luskutoff, D.J., J. Cell. Physiol., 96, 361 (1978).

110. Biggs, R., (editor) The Preparation of General Reagents and Coagulation Factors in Human Blood Coagulation, Haemostasis and Thrombosis, 2nd edition, Blackwell Scientific Publications (1976).

111. Kawaoi, A., Uchida, T., et al, Cell Structure and Function (Japan), 2, 297 (1977).

112. Lash, J.W., Burger, M.M. (editors), Cell and Tissue Interactions, Raven Press, p. 57 (1977).

113. Erickson, C.A., Trinkaus, J.P., Exp. Cell Res. 99, 375 (1976).

114. Goldfarb, R.H., Quigley, J.P. Cancer Res., 38, 4601 (1978).

115. Wilson, E.L., Reich, E., Cell, 15, 385 (1978).

116. Quigley, J.P., Cell, 17, 131 (1979).

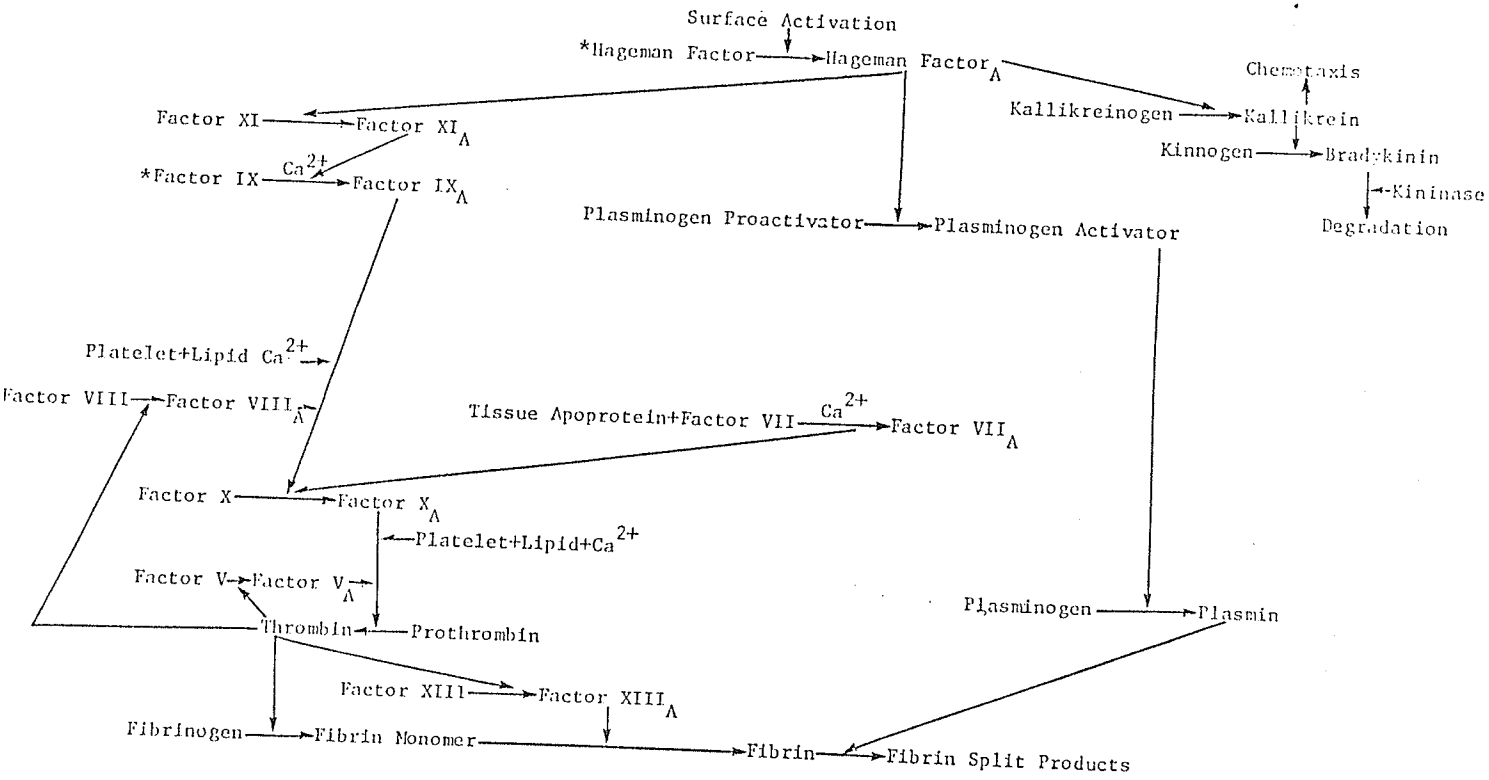
117. E. Kroeger, Personnel Communication, (1979).

118. Hidako, H., Asano, M. et al J. Pharmacol, Exp. Ther. 207, 8 (1978).

APPENDICES

APPENDIX A

MAMMALIAN HAGEMAN FACTOR-DEPENDENT PATHWAYS



A = Activated

* = Reduced amount or absent in chickens

References: 77,78,79,80,81 and 110.

APPENDIX B: Trypsinization

Trypsin: Grand Island Biological, Grand Island, New York. Trypsin 2.5% (10 X), 25 grams trypsin (1:250)/ℓ normal saline. Porcine parvovirus tested.

1. <u>10X PEG-CMF (Component of PM4-CF and PEG-CF medium)</u>

10X PEG-CMF: Physiological Electrolytes and Glucose-Calcium Magnesium Free ΔNaCl compensated.

For 1 litre preparation weigh out:

NaCl (final total 6800 mg/litre)	62.2g
$NaHCO_3$ (final total 2200 mg/litre)	22.0g
Glucose (final 2500 mg/litre)	25.0g
KCl (final 4.5 mM=336 mg/litre)	3.4g
NaH_2PO_4 H_2O (final 3.0 mg% inorganic phosphorus-134 mg/litre)	1.3g

Weigh these out into a 1 litre beaker and dissolve in 800 ml of sterile glass distilled (g.d.) H_2O and bring up to final volume 1000 ml in a 1000 ml graduated cylinder. The medium is then sterilized using an all-glass vacuum filter apparatus (sterile) (47 mm diameter size filter) using a 0.45um Millipore filter. After filtration the sterile 10X PEG-CMF is poured into a sterile Nalgene bottle and equilibrated with CO_2 gas at 10 psi for 5 minutes and stored in the refrigerator ($4^{\circ}C$).

2. PEG-CF (Electrolytes and Glucose for Wash and Trypsinization of Cells)

PEG-CF: Physiological Electrolytes and Glucose-Calcium Free Medium for 1 litre preparation: Add 100 ml 10X PEG-CF to 893 ml d.d. sterile H_2O and then add 6.8 ml 100 mM $MgSO_4.7\ H_2O$.

3. Trypsinization of cells: 0.025% trypsin; trypsin + PEG-CF.

APPENDIX C: Preparation Synthetic Medium (SC 6)

Concentrate Preparations

1. 100X Lactic acid, Na pyruvate, Na citerate, Glycerol.

Lactic acid (final 100 mg/litre)	10g
Na pyruvate (final 12 mg/litre)	1.2g
Na citerate (final 25 mg/litre)	2.5g
Glycerol (final 14 mg/litre)	1.1g

Dissolve above in 1000ml of glass distilled H_2O and sterilize using a sterile vacuum filter apparatus with a 0.45um Millipore filter. The solution is frozen until needed.

2. 100X Amino acids in HCl.

Amino Acids	Final Concentration mg/litre	Grams Weighed Out Into 1 Litre Graduated Beaker
Arginine	15	1.5
Alanine	35	3.5
Cystine	12	1.2
Glutamic Acid	7	0.7
Glycine	16	1.6
Histidine	12	1.2
Isoleucine	9	0.9
Leucine	17	1.7
Lysine	28	2.8
Methionine	4	0.4
Phenylalanine	9	0.9
Proline	24	2.4
Serine	12	2.4
Threonine	15	1.5
Tyrosine	10	1.0
Valine	30	3.0

Add 500ml 2N HCl* to above amino acids, dissolve with a magnetic spinbar. Bring solution value up to 1000ml mark in a graduated cylinder with glass distilled H_2O. Sterilize

this solution as in part 1 and then freeze until needed.

*2N HCl: Add 200ml concentrated HCl (36% by weight, 11.6M) to 960ml glass distilled H_2O.

3. 100X Physiological Glutamine, Asparagine and Tryptophan.

Amino Acids	Final Concentration mg/litre	Grams Weighed Out Into 1 Litre Graduated Beaker
Glutamine	83	8.3
Asparagine	6	0.6
Tryptophan	2	0.2

Dissolve the above to a final value of litre glass distilled H_2O and sterilize by methods used in part 1. Freeze until needed.

4. 1000X Physiological H_2O - Soluble Vitamins +NH_4Cl (S folate, ASA).

Weigh out the following:

Thiamine.HCl (final 0.1 mg/litre)	100 mg
Pyridoxal'HCl (final 0.005 mg/litre)	50 mg
Nicotinamide (fin al 0.1 mg/litre)	100 mg
Riboflavin (final 0.01 mg/litre	10 mg
d-Biotin (final 0.01 mg/litre)	10 mg
Ca^{2+} Pantothenate (final 0.15 mg/litre)	150 mg
Choline Chloride (final 1.5 mg/litre)	1.5 mg
Inositol (final 5 mg/litre)	5.0 mg
NH_4Cl (final 1.2 mg/litre)	1.2 g

Dissolve above in 1 litre of glass distilled H_2O and sterilize by methods used in part 1, then freeze until needed.

5. 1000X Ampicillin: Antibiotic (Bristol-Myers, Quebec)
Dissolve Na Ampicillin (final 100 mg/litre) 2 grams in 20 ml of glass distilled H_2O and sterilize by methods used in part A, then freeze until needed.

6. 100X $MgSO_4 \cdot H_2O$ ($MgSO_4 \cdot 7H_2O$, final 0.68 mM)
Dissolve 24.7 grams $MgSO_4 \cdot 7H_2O$ in 1000 ml glass distilled H_2O, sterilize by methods used in part A and store in refrigerator.

7. 1 N NaOH
Dissolve 40 grams NaOH in glass distilled H_2O and dilute to 1000 ml, then sterilize by methods used in part A.

8. 1000X Folinic acid 0.020 mg/litre
Dissolve 20 mg folinic acid in 10 ml glass distilled H_2O and sterilize by methods used in part A (equivalent to 10^5 x 0.020 mg/litre). Dilute this 10^{-2} with PEG-CF to give 1000X folinic acid 0.020 mg/litre. Freeze solution until needed.

9. 100 mM $CaCl_2$
Dissolve 11.1 grams $CaCl_2$ in 1000 ml glass distilled H_2O, sterilize by methods in part A, then store in refrigerator.

APPENDIX C: Preparation of PM4-CF (Folate, Ca^{2+} Free Medium

Concentrate Preparations

This procedure is done in a sterile calibrated 4l Erlenmyer flask with approximately 3200ml sterile glass distilled H_2O. Add the following:

10X PEG-CMF Na compensated	400ml
100X Lactic acid, Na pyruvate, Na Citerate, Glycerol	40ml
100X Amino acids in HCl	40ml
100X Glutamine, Asparagine, Tryptophan	40ml
1000X Physiological water-soluble vitamins ($\bar{S}$ folate, ASA)	4ml
1000X Ampicillin	4ml
100X $MgSO_4 \cdot 7H_2O$	27.2ml
1 N NaOH	40ml

Bring up to 4000 ml on flask with glass distilled H_2O. This solution is referred to as PM4-CF. To convert PM4-CF to SC6-CF do the following: Add to 1 litre PM4-CF, 1 ml 1000X folinic acid 0.020 mg/litre. This synthesis medium is known as SC6-CF. To convert 1 litre SC6-CF to 1 litre SC6 and 12 ml 100 mM $CaCl_2$. This brings the total Ca^{2+} in the synthetic medium to 1.2 mM.

APPENDIX D: Complete Count Solution

Media Preparation

1. Trypsinizing solution used when preparing cells for counting. Prepare by combining 100 ml PBS-CF, 4 ml clarified 2.5% trypsin 1 ml 10% w/v sodium azide* and 0.2 ml antifoam AF (Dow Corning Corporation, Midland, Michigan) 1:100.

*Sodium azide 10% w/v:10 grams Na azide dissolved in 100ml glass distilled H_2O.

2. $CaCl_2$ 20X

Combine 7.5 ml 100mM $CaCl_2$ with 17.5 ml H_2O.