ABSTRACT

STUDIES ON THE VIRAL ETIOLOGY OF VERRUCA VULGARIS
IN TISSUE CELL CULTURE

By Hajime Hayashi

Clinical observations and early human injection experiments have shown that verruca vulgaris (common human wart) was the result of infection by a virus. Although many attempts have been made, the agent has not been recovered in tissue cell cultures.

Over 200 samples of verruca tissues from 195 patients were tested in 13 different tissue cell lines. Only when cells from verruca tissue were placed in contact with the AU cell cultures was cytopathic effect observed. Cell-free material from verruca tissues or cell cultures failed to produce visible cytopathic effect in vitro. Subcultures of three strains of the agent from three different patients were maintained through 23, 26, and 32 serial passages, showing that the agent could be propagated by cell to cell transfer. Gamma globulin and homologous sera, when used undiluted in a cell-serum neutralization test prevented cell to cell transfer of the agent. Dilution of the sera or globulin did not prevent infection indicating that large amounts of antibodies were necessary to prevent the agent from infecting normal cells.

Many attempts were made to establish a new epithelial cell line from normal human skin and verruca tissues but none was successful.

Because of the hazards involved in injecting humans, an attempt was made to establish in animals the agent isolated in tissue cell cultures. Monkeys, rabbits, hamsters, mice, and chickens were injected

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1961

This work is

respectfully dedicated

to

MY FIANCEE AND PARENTS

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. Walter H. Hack, without whose constant interest, patient understanding, and willingness to help, this investigation would not have been possible.

Appreciation is extended to Dr. W. L. Hallmann, Dr. E. D. Devereux, Dr. J. J. Stockton, Dr. J. L. Fairley, and Dr. R. L. Anderson for their helpful suggestions and criticisms of this thesis.

Thanks are also due to the American Cancer Society whose financial assistance enabled the author to hold the position of graduate research assistant.

Sincere thanks are expressed to Miss Joyce A. Remsberg, Mrs. Gail D. Riegle, Mrs. Janet W. Russell, and other workers for their cooperation and technical assistance.

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INTRODUCTION

The theory that viruses might cause tumors was originally proposed by Borrel (1903). Although his attempts to discover the cause of tumors were unsuccessful, he suspected that tumors were the result of viruses infecting the cells. The discovery of the viruses of leukosis by Ellermann and Bang (1908) and sarcoma by Rous (1911) stimulated a search for tumor-inducing viruses. These initial studies were very fruitful for several years and much of the fundamental knowledge we have today, regarding virus-induced tumors, came from this early work.

Recently Dmochowski (1961) summarized that within the past ten years 150 viruses have been isolated, many of which have been found responsible for various types of tumors in animals.

Although many viruses in animals have been isolated as causative agents of tumors, no proven tumor-producing agent of man has been isolated. Many agents have been isolated from man, especially from leukemia, but it is not known if these agents are the cause of leukemia. Man is not a satisfactory experimental animal.

The present literature states that the cause of human warts (verruca vulgaris) is a filterable agent. This stems from the very early work of Ciuffo (1907) who ground up wart tissue, filtered the extract and inoculated himself with the filtrate. Many attempts have been made since 1907 to cultivate the agent of verruca but none were successful.

The experiences in this thesis were those dealing with the many attempts to isolate an etiological agent from human wart tissues. The methods employed were many but centered around cell cultures, the newest tool for the study of viruses. Experimental animals were inoculated by

many routes, employing known "tricks of the trade". Tissue extracts and fluid phase nutrients from cell cultures were studied under the electron microscope in the search for this agent.

Any agent isolated from wart tissues would have great value as a model antigen. The agent would open the door to many unknown questions about virus-induced tumors and especially the virus-tissue relationship.

I. Perspectives in viral etiology of tumors

A neoplasm is an independent overgrowth of tissue which serves no useful purpose and is usually destructive to normal tissues. The term tumor is properly applied to any neoplasm, benign or malignant.

Malignant tumors are those that endanger life. They infiltrate and destroy the tissue locally and metastasize to distant tissues. The malignant cells are undifferentiated or embryonic. Benign tumors are composed of well-differentiated tissues which approach or attain the structure of adult tissues. They do not spread widely throughout the body, grow so rapidly, or kill so regularly as malignant tumors. The word cancer is used in a popular sense to designate any malignant tumor.

Methods of investigating tumor-inducing viruses do not differ from those used in the study of other infective agents. These same biological and immunological techniques have been used since the beginning of this century.

When an infectious process was studied and the etiological agent was not observed under the microscope, the infection was thereafter considered as a virus infection. The inoculation of animals with this infectious material sometimes resulted in the transmission of the infection and serial transfers were accomplished. The discovery of various types of filters that would retain bacteria and permit smaller agents to pass

through into the filtrate soon demonstrated viruses.

Ciuffo (1907) reported that the Berkefeld N filtrate of wart material from man was infectious and demonstrated the long interval between the time of injection and the appearance of warts on his own arm. In the following year Ellermann and Bang (1908) observed that when blood, emulsions of organs or filtrates of organs from leukemic chickens were inoculated into healthy birds, fowl leukemia developed in the experimental birds. Rous (1911) discovered the transmission of sarcoma in chickens by injecting cell-free filtrates into experimental chickens. The following year Rous et al. (1912) demonstrated that osteochondro-sarcoma was a virus-induced disease of birds. Claude and Murphy (1933) reviewed the reports of virus-induced tumors of chickens and included myxosarcoma isolated by Fujinami and Inamoto in 1910, the spindle-cell sarcoma by Lange in 1914, the fibromyxosarcoma by Hayashi in 1915 and the endothelioma by Begg in 1927.

The successful isolations of agents from chickens prompted a search for the causative agent in tumors from other animals. Oral papillomatosis of dogs has the characteristics of warts in man. De Monbreun and Goodpasteur (1932) removed the warts from a dog and, after extracting the tissue, filtered the fluid phase through a Berkefeld filter. With this cell-free filtrate they were able to produce warts in puppies but not in rabbits, rats, mice, guinea pigs, kittens nor monkeys.

Shope (1932a) upon examining a cottontail rabbit, discovered a fibroma-like tumor. After the tumor had been minced and extracted with saline solution, rabbits were injected with the extract, and tumors were produced. Shope (1932b) later used cell-free extracts to reproduce this tumor in rabbits and demonstrated the relationship of the fibroma and

myxomatosis agents of rabbits.

One of the many peculiarities of host susceptibility was demonstrated by Rous and Beard (1935). They observed that, when rabbit papilloma virus was introduced into wild cottontail rabbits, the tumors grew but many regressed with time. In the cottontail rabbits the disease was a benign infection. When the tumor material was removed from the cottontail rabbits and the virus extracted from the tissues and then injected into domestic rabbits, the result was malignant infection.

Bittner (1936) was the first to demonstrate that carcinoma tissue contained a transmissible agent. The agent was isolated from the mammary glands of mice, and filtrates from infected tissues readily infected experimentally injected mice. It was also shown that infection took place naturally between mother and offspring through the milk. Isolation of the Bittner mammary virus led to the speculation that in man cancer infection takes place in new born and only manifests itself in later life. This theory has not been proved for man, nor under these circumstances can we directly compare man and mouse. Support of this theory was strenghthened by the isolation of "mouse poliovirus" or Theiler's virus (1937) in mouse colonies. The agent was a latent virus frequently found in experimental mouse colonies. Although the virus was latent in most of the individuals, approximately one per cent of the animals developed paralysis and died from the infection. The virus was transmitted to the young through contact with adults in the colony. Theiler's virus was demonstrated as a latent infection by applying certain physiological insults to adult members of the colony.

Mouse leukemia, a virus-induced disease, was discovered by Gross (1951). His success was dependent upon injecting cell-free material from leukemic AK mice into newly born inbred C3H mice. The results of Gross

have been confirmed by several investigators (Furth et al., 1956; Stewart, 1953; Woolley and Small, 1956). Friend (1957a, b) independently observed leukemia in mice and was able to show transmission to new born mice by injecting cell-free splenic extracts. This leukemia virus resulted in myelogenous leukemia while the virus isolated by Gross most often produced lymphocytic leukemia.

In an attempt to apply the knowledge learned through the study of mouse leukemias, tissue extracts from human cases of leukemia have been injected into new born mice. Leukemia has resulted in a small proportion of the injected animals, and only certain strains of mice appear to be susceptible to the agent. The type of leukemia developing in the serial passages can not be predicted.

Certain undisputable facts have resulted in the study of transmissible virus-tumors. 1) The younger the experimental animals used, the greater the possibility of isolation success. 2) Most virus-induced tumors in animals require a much longer "incubation period" than has been experienced with the usual infective-type virus diseases. 3) The resulting tumor produced by injecting a certain tumor-inducing virus can not be predicted. Since tumors are classified by what they look like to the pathologist, the name given to a piece of tumor tissue varies.

The fact remains that viruses are repeatedly recovered from cell-free extracts of tumors from animals and these extracts can produce disease when introduced into the susceptible experimental animal.

The in vitro cultivation of tumor-producing viruses has been accomplished repeatedly for the past thirty years. It was not until recently that tissue cell cultures were extensively used to propagate these agents.

Chicken sarcoma virus was cultivated in vitro by Furth and Stubbs (1934). They placed pieces of chick tissue in plasma clot cultures and, after outgrowth of cells, seeded the cultures with chicken sarcoma virus. In this manner they were able to show multiplication of the virus in the cultures. The plasma clot culture method was used extensively in the cultivation of viruses during this period.

The in vitro cultivation of all types of viruses expanded when Dulbecco and Vogt (1954) discovered that when tissue cells were treated with trypsin and then placed in cultures, a monolayer of cells developed. The cell sheet could be then seeded with virus and the resulting virus multiplication be visually observed by plaque formation.

Evidence of virus multiplication was called the cytopathic effect (CPE) and was applied to tumor inducing virus by Lo et al. (1955). They observed that fragments of normal chicken fibroblasts, when grown in plasma clots, were lysed after seeding the culture with Rous sarcoma virus. The CPE consisted of degeneration of cells and lysis of the clot. Serial subcultures of the nutrient fluid from these cultures produced degeneration of cells, giant cell formation and a variety of cell shapes and sizes. Non-infected control cultures remained uniform.

Mouse leukemia virus was successfully cultivated in monkey kidney monolayer cells by Stewart et al. (1957). The CPE seen in these cultures was not typical as the cells of the culture appeared to pile one adjacent to another. However, fluid from the cultures, when injected into mice, produced leukemia. When mouse embryo cells were used for cultures, Eddy et al. (1958b) found the mouse leukemia agent produced a cytopathic effect. In early passages of the agent the cells did not appear different from those in control cultures but in two or three weeks some differences in

cell morphology did take place. As "blind" passages were continued cytopathic changes included an increase in pyknotic cells until the majority of the cells in the cultures were affected, causing removal of the cells from the wall of the tubes. Finally adaption of the agent to the in vitro culture resulted in subcultures all of which produced cytopathic effect. The agent induced leukemia when injected into mice.

The agent of visceral lymphomatosis was shown to produce cytopathic effect in cell cultures of chicken embryo liver by Sharpless et al. (1958). Fontes et al. (1958) also described cytopathic effect with this agent cultivated in vitro on chicken cells. In both reports, CPE was observed only after several "blind" passages in cultures. The cytopathic effects were characterized by Fontes et al. as refractable granules appearing in the cytoplasm of the polygonal epithelial-like cells; the nucleus became pyknotic and this was followed by shrinkage of the cytoplasm or rounding of the cells. Some cultures contained groups of cells which formed grape-like clusters. The cells finally detached from the tube wall.

Stewart (1953) described the induction of a parotid gland tumor in mice. The mice had been inoculated, within a few hours after birth, with a cell-free extract of tissue from a spontaneous leukemia in a mouse. The methods Stewart used were those described by Gross (1951) for producing leukemia in mice. Stewart's results indicated that the virus she recovered had oncogenic activity and was unique in several properties. The agent called the polyoma virus, has stimulated search for other agents producing tumors in animal and man, and has been extensively used as a model system. The agent and its resulting tumor have the following properties:

1) The tumor inducing agent can be propagated in vitro in tissue

cell cultures (Stewart et al., 1957; Eddy et al., 1958a; Stewart et al., 1958).

2) Its oncogenic activity not only crosses strain barriers in mice (Stewart et al., 1958; Mirand et al., 1958) but also crosses species barriers producing tumors in hamsters (Eddy et al., 1958a; Stewart et al., 1960) and rats (Eddy et al., 1959).

3) It has the properties of many of the usual types of infectious viruses such as high antigenicity (Stewart and Eddy, 1959a; Stewart et al., 1959), production of cytopathic changes in cell cultures (Eddy et al., 1958b) and hemagglutination of erythrocytes from several animal species (Eddy et al., 1958c). The hemagglutination reaction provides a simple method for the titration of viral activity and specific antibodies in sera.

As with many of the tumor-inducing agents, polyoma virus when injected into animals produces more than one type of primary tumor. That is why the agent has been called the polyoma virus (Eddy et al., 1958b). The agent produces malignant tumors and has, in many respects, altered the present concept of what is and what is not malignancy.

The serology of tumor resistance has a complex and unmeaningful background. Animals surviving the tumor infection are resistant to the agent. Serological demonstration of this resistance has presented certain difficulties. Burmester (1955) has shown passive resistance in chickens in vitro and in vivo with the lymphomatosis agent. Sharpless et al. (1958) and Fontes et al. (1958) demonstrated serum neutralization against the lymphomatosis agent in vitro. The antibodies were produced by repeated injection of lymphomatosis virus into chickens. Neutralizing antibodies to the Friend's leukemia agent of mice have been reported by

Friend (1957a, b).

The polyoma virus probably has much to offer as a model in attempting to understand the reaction of host resistance to the tumor agents. This agent is without doubt the most active of the tumor agents with regards to serological tests and antibody formation. Neutralization, hemagglutination inhibition and complement fixation tests can be applied to the polyoma agent and its specific antiserum (Eddy et al., 1958c; Rowe et al., 1958). On the other hand, several agents produce antibodies to tumor cells as well as the agent (Beard et al., 1957; Cheever and Janeway, 1941; Eckert et al., 1955; Gorer and Law, 1949; Kabat and Furth, 1941; Law and Malmgren, 1951 and Malmgren et al., 1951).

Other means of detecting tumor-inducing agents have shown some success. Electron micrographs of ultra-thin sections of tissues or ultracentrifuge concentrates of tissue extracts have resulted in objects which appear to be virus particles in these preparations (Dmochowski, 1959 and 1961). Visual observations of these virus-like particles from tumors or tissues and confirmation that they are the causative agent are lacking. Electron microscopy has its painful limitations when used alone, but when combined with other means of exploration it becomes a valuable asset to the whole understanding of the problems.

The presence of tumor-inducing agents, although poorly understood because of many complicating factors involved, has been proved in many ways. Not all of the viruses producing tumors have been reviewed in this work, however, the following general outline lists those animal tumors which are thought to be produced by viruses.

(1) Avian leukosis complex

   a. Lymphomatosis

   b. Myeloblastosis

c. Erythroblastosis

(2) Avian sarcoma

(3) Amphibian renal carcinoma

(4) Mammalian papillomatosis

a. In man

b. In dogs

c. In rabbits

d. In cattle etc.

(5) Mouse mammary carcinoma

(6) Mouse leukemia

a. Lymphocytic

b. Myelogenous

Recently Stewart and Irwin (1960) described proliferative changes in certain cell cultures inoculated with five specimens taken from patients having tumors. The first was a surgical specimen from a papillomatosis growth of the tongue in a 73-year-old male. The second specimen was from tissues of a 22-month-old child with an embryonal tumor. The next was urine from a 6-year-old boy with a renal neuroblastoma and the last specimen was from an 8-year-old child with Hodgkin's disease. Extracts from all of these specimens were placed in contact with cell cultures of human tissues and "blind" passages were made. It was noted that after 3-4 passages over 4-5 months, focal areas with abnormal migration of the cells were observed in the inoculated cultures. The cultures were transferred. Although proliferative effect was reproducible in tissue cultures by serial passage of the culture fluids, it appeared that cells or cell particles were necessary for transfer, and culture activity was lost by filtration or centrifugation of the tissue culture fluid. Transfer of

cells instead of nutrient fluid is new to tissue culture propagation of viruses.

Weller et al. (1958) were able to propagate varicella and herpes zoster in tissue cultures only if infected tissues were placed in contact with the cultured cells. Passage also required transmission of infected cells from culture to culture.

Rowe et al. (1957) was able to propagate the human salivary gland virus in cultures of human skin only when infected tissue was used as inoculum and transfer material contained infected cells. It appears that in some cases the parasite is closely associated with the cell and infection takes place in vitro from infected cell to "normal" cells.

II. The etiology of verruca vulgaris

Verrucae, or common human warts, are classified (Anderson, 1957) as benign tumors, representing a thickening or projection of epidermis. Several adjectives are used to describe verrucae depending upon their shape, location or the clinical features of the lesion. Verruca vulgaris, plantaris, digitala, filiformis, juvenilis and senilis are all warts and probably are all caused by a common etiological agent.

Verruca vulgaris is most commonly seen on children and is found on fingers, hands, arms and legs or generally those areas of the body not clothed. They occur singly or in groups. Histologically, verruca vulgaris is characterized by a papillary acanthosis surmounted by friable keratic material. The cells of the stratum granulosum are often acidophilic and vacuolated. A loose infiltration of various mononuclear cells may be present in the papillae (Anderson, 1957).

The infective nature of warts has no dated record. People knew that

warts could be spread by contact and history has many amusing stories about this strange condition. The cause and cure of warts also have much history in the development of man. In ancient times as well as today, persons were identified by their warts. The exact cause of warts has interested many people but the first recorded experiments regarding their etiology was in 1896 when Jadassohn (1896) published his work, beginning in 1894, on the cause and transmission of warts. Jadassohn removed wart tissue from his patients and after grinding up the tissue in salt solution or by using small fragments of tissue, injected the skin of his own arms and hands with the specimen. His work extended to experiments on colleagues, and 74 inoculations resulted in 33 reproductions of warts. Jadassohn's experiments settled the question regarding the infectious nature of the wart, but he made no attempt to explain the etiological factor involved. In 1907, Ciuffo (1907) produced warts by injecting Berkefeld N filtrates of extracted wart tissue. He inoculated the patient from whom the wart was originally removed and the skin of his own hand. After 5 months incubation period, warts appeared in both subjects. Not only did Ciuffo confirm Jadassohn's original observations but he demonstrated that a filtrate of wart material was infectious and that tissue cells and parasites were excluded from the inoculum. Ciuffo's work has been repeatedly referred to regarding the viral etiology of human warts. The filterability of the wart agent was confirmed by Serra in 1908. In 1919 Wile and Kingery (1919) filtered the extracts of warts and reproduced warts with these filtrates. Kingery (1921) produced a second generation by removing an experimentally produced wart and after filtering the extract, reinoculated a volunteer, producing again a wart growth. In all of these transmission experiments in human

beings, a prolonged incubation period of 6 months or more was observed before the appearance of the wart growth. In most of the experiments, the warts regressed shortly after their appearance.

The cause of warts was not discussed again until 1953 when Bivins (1953), a poultry pathologist, removed a wart from his finger and seeded an extract of the wart upon the chorioallantoic membrane of a developing chick embryo. Resulting plaques on the membrane were misinterpreted by Bivins to be the result of wart virus. Siegel (1956) found that the agent isolated by Bivins was a strain of avian pox virus.

Strauss et al. (1949, 1950) examined concentrated extracts of wart tissues under the electron microscope. They found crystalline particles which they considered to be virus particles in many of their preparations. The particles in the crystalline array averaged 52 mu in diameter but averaged 68 mu when not observed in the crystalline-like clusters. The observations of Strauss et al. were not confirmed by Siegel (1960). The latter used a refined concentration method and found a large variety of particle sizes, but some preparations contained a 16 mu virus-like particle which Siegel considered more uniform and suggested might be the wart agent.

The etiological agent of human warts has been considered to be virus since 1907 when Ciuffo filtered the wart tissue extract and reproduced warts by injecting the filtrate into the skin of his hand. The viruses producing warts in dogs (De Monbreun and Goodpasture, 1932), cattle (Olson et al., 1959) and deer (Shope et al., 1958) have been characterized to some extent because experimental animals were available. The results presented here are efforts to isolate the human wart agent by tissue culture methods.

Throughout this thesis the word "agent" has been used synonymous with "virus" although the wart agent as described herein does not fit the classical criterion of a virus.

MATERIALS AND METHODS

Many of the reagents and composition of the reagents were altered during the three years these experiments were conducted. Whenever standard or stock reagents proved satisfactory their use was continued but many times the compositions were changed to meet the needs of the work in progress. The various solutions of growth and maintenance media are given here as stock solutions and when altered the changes will be given in the results of that particular set of experiments. All reagents used were diluted with water which had been freshly distilled through three cycles in an all glass distilling apparatus.

Tissue cell cultures were obtained, whenever possible, from commercial sources. When not commercially available, the cultures were obtained from the best source available, generally the author describing a certain cell line. In the latter case, the culture was sent to the laboratory by air and thereafter the culture was propagated in the laboratory. In table 1, the cell lines used and their sources are listed.

Reagents

1. Hanks' balanced salt solution (BSS, Hanks and Wallace, 1949)

   Solution 1: Dissolve 1.4 g $CaCl_2$ in 200 ml distilled water.

   Solution 2: Dissolve the following in 900 ml of distilled water.

Glucose	10 grams	KH_2PO_4	0.6 gram
NaCl	80 "	$Na_2HPO_4.2H_2O$	0.6 "
KCl	4 "	Phenol red	0.2 "
$MgSO_4.7H_2O$	2 "		

The two solutions were separately autoclaved at 10 lbs pressure (115 C) for 10 min. The solutions were mixed together and approximately 0.35 g $NaHCO_3$ was added to adjust the pH to 7.2.

Table 1: Origin of cell cultures used.

Name	Tissue	Origin	Medium Required	Cells Obtained From
Human conjunctiva	Normal human* conjunctiva	Chang	BME** & 10% H.S.x	Tuskegee Inst., Alabama
AU	Normal human* skin	Wheeler et al.	BME or YEM** & 20% H.S.	Wheeler, Univ. of Virginia, Va.
HuS 3075	Normal human* skin	Perry et al.	NCTC 109 & 10% A.S.xx	Natl. Cancer Inst., NIH & Tissue Bank, Natl. Med. Center
Human intestine	Jejunum of* human embryo	Henle	BME & 10% H.S.	Microbiol. Assoc., Bethesda, Md.
Human heart	Human adult* heart	Girardi	BME & 10% H.S.	Microbiol. Assoc., Bethesda, Md.
HeLa	Carcinoma of* human cervix	Gey	BME & 10% A.S.	Microbiol. Assoc., Bethesda, Med.
T-1	Human kidney & lung reticulo-sarcoma*	van der Veen	199** & 5% A.S.	Pitman Moore Co., Zionsville, Ind.
Monkey kidney	Epithelium, primary culture		199 & 5% A.S.	Microbiol. Assoc., Bethesda, Md.
MAF	Human embryonic skin and muscle		BME & 10% H.S.	Microbiol. Assoc., Bethesda, Md.
D-189	"Malignant trans-* formation of human foreskin"	Leighton	BME & 10% H. S.	Microbiol. Assoc., Bethesda, Md.
Detroit-98	Human adult* sternal marrow	Berman and Stulberg	BME & 10% H.S.	Microbiol. Assoc., Bethesda, Md.
Human amnion	Epithelium, primary culture		BME & 10% H.S.	Payne, Univ. of Mich., Mich.
Chick liver	Embryonic chick liver		199 & 5% A.S.	Dept. of Microbiol., Mich. State Univ.

* Cell lines propagated in this laboratory.

** See code for abbreviations under reagents.

x H.S. = Human serum.

xx A.S. = Animal serum.

2. Basal medium, Eagle (BME, Eagle, 1955)

To 98 ml of Hanks' balanced salt solution, 1 ml of vitamin mixture (100x) and 1 ml of amino acid mixture (100x) were added. The vitamin and amino acid mixtures consisted of the following chemicals and each was dissolved in 1 liter of distilled water:

Vitamin mixture		Amino acid mixture	
Biotin	0.1 g	Arginine	2.1 g
Choline	0.1 "	Cystine	1.2 "
Folic acid	0.1 "	Histidine	0.8 "
Nicotinamide	0.1 "	Isoleucine	2.6 "
Pantothenic acid	0.1 "	Leucine	2.6 "
Pyridoxal	0.1 "	Lysine	2.6 "
Thiamin	0.1 "	Methionine	0.8 "
Riboflavin	0.01 "	Phenylalanine	1.6 "
i-Inositol.$2H_2O$	0.18 "	Threonine	2.4 "
		Tryptophan	0.4 "
		Tyrosine	1.8 "
		Valine	2.4 "
		Glutamine	30.0 "

3. Yeast extract medium (YEM, Robertson et al., 1955)

To Hanks' balanced salt solution add 0.1 per cent yeast extract (Difco) and 0.35 per cent glucose.

4. Mixture 199 (Morgan et al., 1950)

This medium was obtained as a commercially prepared reagent and consisted of the following chemicals dissolved in 1 liter of distilled water:

DL-Tryptophane	20 mg	L-Histidine	20 mg
DL-Phenylalanine	50 "	L-Lysine	70 "
DL-Isoleucine	40 "	Sodium acetate	50 "
DL-Leucine	120 "	DL-Aspartic acid	60 "
DL-Methionine	30 "	DL-Glutamic acid	150 "
DL-Serine	50 "	L-Tyrosine	40 "
DL-Threonine	60 "	L-Cystine	20 "
DL-Valine	50 "	Adenine HCl	10 "
DL-α-Alanine	50 "	Guanine HCl	0.30 "
L-Proline	40 "	Cysteine HCl	10 "
L-Hydroxyproline	10 "	Xanthine	0.30 "
Aminoacetic acid	50 "	Hypoxanthine	0.30 "
L-Argine	70 "	Thymine	0.30 "

Uracil	0.30 mg	Folic acid	0.01 mg
Vitamin A	10 "	L-Ascorbic acid	5.0 "
Calciferol	0.10 "	Glutathione	5.0 "
Cholesterol	0.20 "	D-Ribose	0.50 "
Menadion	0.01 "	Adenylic acid	0.20 "
Disodium α-tocopherol phosphate	0.01 "	Adenosine triphosphate.	0.010 "
Niacine	0.025"	L-Glutamine	100 "
Niacinamide	0.025"	Ferric nitrate	0.10 "
Pyridoxine HCl	0.025"	NaCl	8000 "
Pyridoxal HCl	0.025"	KCl	400 "
Thiamin HCl	0.010"	$CaCl_2$	140 "
Riboflavin	0.010"	$MgSO_4.7H_2O$	100 "
Calcium pantothenate	0.010"	$MgCl_2.6H_2O$	100 "
L-Inositol	0.050"	$Na_2HPO_4.2H_2O$	60 "
p-Aminobenzoic acid	0.050"	KH_2PO_4	60 "
Choline chloride	0.500"	Glucose	1000 "
D-Biotin	0.01 "	Phenol red	20 "

The final pH was adjusted to 7.2 with $NaHCO_3$ just prior to use.

4. NCTC 109 (McQuilkin et al., 1957)

Purchased from Microbiological Assoc., Bethesda, Md. and consisted of the following chemicals dissolved in 1 liter of distilled water:

L-Alanine	0.3148 mg	Thiamin HCl	0.00025 mg
L-α-Amino-n-butyric acid	0.0551 "	Riboflavin	0.00025 "
L-Arginine	0.2576 "	Pyridoxine HCl	0.000625"
L-Asparagine	0.0809 "	Pyridoxal HCl	0.000625"
L-Aspartic acid	0.0991 "	Niacin	0.000625"
L-Cystine	0.1049 "	Niacinamide	0.000625"
D-Glucosamine	0.0320 "	Calcium pantothenate	0.00025 "
L-Glutamic acid	0.0826 "	Biotin	0.00025 "
L-Glutamine	1.3573 "	Folic acid	0.00025 "
Glycine	0.1351 "	Choline chloride	0.0125 "
L-Histidine	0.1973 "	i-Inositol	0.00125 "
Hydroxy-L-Proline	0.0409 "	p-Aminobenzoic acid	0.00125 "
L-Isoleucine	0.1804 "	Cyanocobalmin	0.1 "
L-Leucine	0.2044 "	Vitamin A	0.0025 "
L-Lysine	0.3075 "	Calciferol	0.0025 "
L-Methionine	0.0444 "	Menadion	0.00025 "
L-Ornithine	0.0738 "	α-Tocopherol phosphate	0.00025 "
L-Phenylalanine	0.1653 "	Glutathione	0.1 "
L-Proline	0.0613 "	Ascorbic acid	0.5 "
L-Serine	0.1075 "	Cysteine HCl	2.6 "
L-Taurine	0.0418 "	Diphosphopyridine nucleotide	0.070 "
L-Threonine	0.1893 "	Triphosphopyridine nucleotide	0.010 "
L-Tryptophan	0.1750 "	Coenzyme A	0.025 "
L-Tyrosine	0.1644 "	Cocarboxylase	0.010 "
L-Valine	0.2500 "		

Flavin adenine dinucleotide	0.010 mg	Sodium acetate	0.5 mg
Uridine triphosphate	0.010 "	NaCl	68.0 "
Deoxyadenosine	0.1 "	KCl	4.0 "
Deoxycytidine HCl	0.1 "	$CaCl_2$	2.0 "
Deoxyguanosine	0.1 "	$MgSO_4.7H_2O$	1.0 "
Thymidine	0.1 "	NaH_2PO_4	1.4 "
5-Methylcytosine	0.001 "	$NaHCO_3$	22.0 "
Glucuronolactone	0.018 "	Dextrose	10.0 "
Sodium glucuronate	0.018 "	Tween 80	0.125 "
		Phenol red	0.2 "

The final pH was already adjusted.

5. Trypsin solution

Trypsin was used to separate cells from tissue or removing cells from the walls of tissue culture tubes. The concentration of trypsin used varied with its use. Usually a concentration of 0.25 g of trypsin (1:250, Difco) in 100 ml calcium free Hanks' solution was used (See solution 2 above in Hanks' balanced salt solution). The trypsin solution was sterilized by Seitz filtration. A 1 per cent solution was used to separate cells from tissues.

6. Antibiotic solution

Penicillin and streptomycin were incorporated into all nutrient fluids used for tissue cultures. Usually 100 units of penicillin and 100 ug of streptomycin per ml of nutrient fluid were employed. This concentration was increased when heavily contaminated tissues were encountered.

7. Chick embryo extract

Fertile chicken eggs were incubated for 9 days. Only those embryos appearing live and well developed upon candling were used. The embryos were removed from the egg, washed in BSS and transferred to the barrel of a 20 cc syringe fitted with a circular stainless steel screen. Pressure was applied to the plunger of the syringe forcing the tissue out of the barrel and into an equal volume of BSS. The extract was then cen-

trifuged at 2500 r.p.m. for 20 minutes, placed in tubes and stored at -20 C until use. Just prior to use the tube was quickly thawed and the fluid again centrifuged at 2500 r.p.m. to remove any formed sediment. The supernatant fluid was used to form serum clots or as nutrient for cell cultures.

Tissues

Wart tissues were removed by total enucleation (Ulbrich et al., 1957). Tissues removed at the dermatologists' offices were placed in sterile vials and either refrigerated at 4 C or frozen in freezing compartments of refrigerators. For shipment to the laboratory the vials were placed in contact with dry ice.

Specimens of warts were also removed in the laboratory. The tissues removed were placed in Hanks' balanced salt solution containing antibiotics and held at 4 C for 24-48 hours. When needed normal skin was removed. Depending upon their use, the specimens were prepared in various ways. Cell-free extracts were prepared by grinding the wart tissues in a mortar with a pestle using alundum as abrasive. Minimum amounts (5 per cent wart suspension) of basal salt solution containing antibiotics, were used as diluent. The suspensions were centrifuged in a horizontal position at 1500 r.p.m. for 10 minutes to remove the cells and debris. Fragments of wart tissues were prepared by mincing the tissue as fine as possible with a blade after the tissue had been stored overnight in Hanks' balanced salt solution and antibiotics. Trypsinized wart tissues were prepared by placing the tissue in one per cent trypsin solution at 4 C for several hours. The cells were separated from the trypsin solution by slow speed centrifugation.

Sterility tests

Sterility control of all tissue culture material was accomplished by seeding sample specimens into several screw capped tubes containing brain heart infusion broth (Difco). The tubes were incubated at 37 C and examined each 24 hours for bacterial growth. Tissue samples were considered free from bacteria only if no growth occurred in the broth after 72 hours incubation. Occasionally, tissue cell cultures were tested for the pleuropneumonia-like organisms by seeding samples of cell cultures, including cells, into special pleuropneumonia medium (Difco).

Tissue other than wart tissue

Many attempts were made to establish a tissue cell line from normal skin. Normal human skin was obtained on demand from John Dunkel, M. D., pathologist, Edward Sparrow Hospital, Lansing, Michigan. Foreskins from new born infants were collected daily. Skin from adults was obtained upon demand at the hospital or removed from volunteers in the laboratory. In experiments using human normal skin, the skin was placed in Hanks' balanced salt solution containing antibiotics and stored at 4 C for 24 hours or until the sterility control tests indicated bacteria were not present in the specimen.

Blood and blood products

Blood serum was used to supplement nutrient solutions for tissue cultures. Sera from various animals were used depending upon the type of cell cultures and whether the cultures were being propagated or maintained. Human blood was obtained through Joseph Venier, M. D., Lansing Red Cross Blood Bank. Whole blood was collected from volunteers, the clot was allowed to form over night and the cells were separated from the

serum by centrifugation. Strict sterility tests were made by placing 0.1 ml of each serum sample into multiple tubes of brain heart infusion broth. Any sample found contaminated was discarded.

Blood was drawn from horses or calves when needed and the serum and cells separated as described for human blood.

Fractionated human blood samples were supplied by the Michigan department of health laboratories, Lansing, Michigan.

Experimental animals

White Swiss mice, Webster's virus susceptible, were reared in the laboratory. Pregnant females were observed daily for birth of their young. Litters were sometimes pooled to obtain sufficient numbers of animals. Adult hamsters were purchased and laboratory bred. Upon birth of the young, the day old hamsters were used in the experiments. Chickens used were hatched from eggs in the laboratory. After hatching, chicks were removed from the incubator, inoculated and placed in heated cages. Half-grown white laboratory rabbits were injected with wart tissues, tissue culture fluids and cells. No attempt was made to use day old rabbits. The monkeys used were Macaca cynamolgus and purchased from animal dealers. The ages of the monkeys were unknown but all had deciduous teeth. X-ray "tanning" of the skin was accomplished by irradiation of the abdomen at 50 cm in 6 doses at 48 hour intervals until 1000 r. had been accumulated.

Electron microscope observations

Extracts were made from wart tissues and normal and infected cell cultures. The method has been described by Strauss et al. (1949, 1950). The extracted material, after slow speed centrifugation to remove debris,

was centrifuged at 6000 r.p.m. for 45 to 60 minutes in a refrigerated International Multispeed centrifuge. The sediment was resuspended in small amounts of distilled water. Minute amounts of the resuspended sediment were placed upon electron microscope collodion prepared screens and after drying were shadow-casted with tungsten oxide before examination.

RESULTS

I. Tissue cell culture studies

A. Inoculation of wart material into tissue cell cultures

The early attempts to isolate the wart agent were made by standard virus-tissue culture methods. The frozen wart tissue was extracted by grinding the tissues in a mortar and diluting the pulp with basal salt solution. After centrifugation to remove the debris, the supernatant fluid was then placed in contact with monolayer tissue cell cultures. Observations were made daily of each culture and any morphology alterations recorded. Any change in cell morphology of any culture was followed by serial "blind" subcultures using the nutrient fluid as passage material. All subcultures were observed for as long as the cells could be maintained. Occasionally morphology changes in cells were seen but consistent results were lacking upon subcultures. In table 2 the results are given for 78 samples of frozen wart samples from 62 different patients.

Two epithelial cell lines were used and the results showed no evidence that the agent was affecting the cells. Subcultures of the nutrient from the human conjunctiva cultures were made at 5 to 14 day intervals (table 3). Seven samples were subcultured twice; one sample was passed 4 times and two samples were passed 5 times but no evidence of agent activity was observed.

Evidence that the causative agent of warts would withstand freezing was lacking, therefore, arrangements were made to have wart tissue removed in the laboratory. Fresh wart material was extracted, treated with trypsin or separated into small fragments prior to placing in contact with the different tissue culture cell lines.

Table 2: Tests on extracts of frozen warts from 62 patients in two tissue cell lines.

Cell Line	No. Samples	Treatment of Wart Tissue	No. Days Observed
Human conjunctiva	66	Extracts	13-21
Human embryonic skin (MAF)	12	Extracts	18-24
Total:	78		

Table 3: The results of subculturing (blind passage) nutrient fluids from human conjunctiva cell cultures

Cell Line	No. Samples	No. Passages	Passage Line	No. Days Observed	Results
Human conjunctiva	7	2	Human conjunctiva	13-14 per passage	Negative
	1	4	Human conjunctiva	13-14 per passage	Negative
	2	5	Human conjunctiva	13-14 per passage	Negative

The results of testing 75 samples of unfrozen wart tissue in eight different cell lines are presented in table 4. All cell cultures were observed for as long as they could be maintained but no evidence of viral activity was observed. In two of these tests (Detroit-98 and D-189 cell lines) the fresh tissue was first extracted and the resulting cell-free extract was frozen. This was necessary because of the difficulty in obtaining the two cell lines at the same time that fresh wart tissues were available. The results given in table 4 represent the wart tissues from 64 patients. In two cell lines, T-1 (human kidney and lung reticulosarcoma) and HuS 3075 (human normal skin), "blind" subcultures were made. The cell free nutrient fluid from previous cultures was used as inoculum. As indicated in table 5, no evidence of agent growth was observed in any of the cultures of any passages. In table 6 the results are given of a "blind" subculturing of combined tissue culture cells in nutrient fluid. The original cultures were inoculated with small (1 mm^3) fragments of wart tissue. After 14 days the cells were mechanically removed from the tube wall and suspended in the nutrient fluid. Both cells and fluid constituted inoculum for subsequent passages. As can be seen, five samples were subcultured three times and observations indicated that there was no visual evidence of agent activity.

In viewing the results given with the various types of tissue cultures used, it was obvious that the agent in frozen or fresh wart material was not easily adapted to <u>in vitro</u> cultivation and a search was made for other cell cultures.

B. <u>Attempts to cultivate primary human skin</u>

As there was no evidence of growth of the wart agent in the epithelial cell cultures, attempts were made to establish a culture of human skin.

Table 4: Tests on fresh wart tissues from 64 patients in eight tissue cell lines.

Cell Lines	No. Samples Tested	Treatment of Wart Tissue	No. Days Observed
Human conjunctiva	8	Extracts	13-21
Human embryonic skin (MAF)	1 7	Trypsinized cells Extracts	14 14-21
Human heart	1 12	Trypsinized cells Extracts	14 14-21
Human intestine	1 10	Trypsinized cells Extracts	21 21
Detroit-98 (Human sternal marrow)	10	Extracted and then frozen	14
D-189 (Malignant transformation of human foreskin)	10	Extracted and then frozen	11
T-1 (Human kidney and reticulosarcoma of lung)	2 2	Pieces Extracts	21 21
Hu3 3075 (Human normal skin)	6 5	Pieces Extracts	14 14
Total:	75		

Table 5: The results of subculturing (blind passage) nutrient fluids from unfrozen wart extracts in two tissue cell lines

Cell Lines	No. Samples	No. Passages	Passage Line	No. Days Observed	Results
T-1 (human kidney & reticulo-sarcoma of lung	2	2	T-1	14	Negative
HuS 3075 (human normal skin)	5	3	HuS 3075	14	Negative

Table 6: The results of subculturing (blind passage) tissue cells and nutrient fluid from unfrozen wart fragment.

Cell Lines	No. Samples	No. Passages	Passage Line	No. Days Observed	Results
HuS 3075 (human normal skin)	5	3	HuS 3075	14 per passage	Negative

The methods used to prepare the skin for cultures were a modification of those described by Perry et al. (1956) and Wheeler et al. (1957). The skin sample was placed into Hanks' balanced salt solution containing antibiotics to free the tissue of viable bacteria. This was followed by permitting the tissue to remain in a one per cent trypsin solution until soft and then transferring to Eagle's basal medium containing five per cent chicken embryo extract and 20 per cent inactivated human serum. After standing for 10-18 hours at 4-5 C, the dermis and epidermis could be separated. The former was then placed into fresh growth medium and shaken to disperse the cells. Horizontal tube cultures were prepared containing approximately 100,000 cells per ml. Eagle's medium with chicken embryo extract and human serum was used for growth medium.

Twenty-four times skin from different individuals was tested in an attempt to establish a skin culture. None of the attempts were successful. The growth obtained was usually fibroblast-like in nature and only occasionally was a monolayer of cells observed. Those cultures surviving two weeks sometimes contained epithelial-like cells and were subcultured. Cultures transferred by trypsinization or mechanical dispersion of the cells failed to grow.

C. Cultivation of wart tissue cells

All attempts, so far, to isolate the causative agent of warts in tissue cell cultures had been fruitless. Attention was then given to the epithelial cells of wart tissues. Attempts were made with 9 different samples to cultivate trypsinized wart cells by the methods described for normal human skin. Only fresh wart tissues were used but the enzyme treatment apparently destroyed the cells.

Small fragments (1 mm^3) of wart tissues were treated with antibiotics in balanced salt solution and then transferred to culture tubes. To attach the tissue fragments to the tube wall, the tissues were allowed to dry slightly prior to the addition of nutrient fluid. The nutrient used was 40 per cent human serum and 5 per cent chicken embryo extract in Hanks' balanced salt solution. The cultures were observed daily. Because of the mass of cells, the nutrients required changing each 24 hours.

The cultures at 24 hours showed an outgrowth of epithelial cells surrounding each tissue fragment but by 48 hours the epithelial cells stopped growing and were released from the tube wall. Figure 1 shows a fragment of tissue with the cells growing out from the edge of the tissue. At this time, fibroblasts were seen to grow out from the tissue in long strands of cells. Figure 2 shows the fibroblast-like sheet of cells after 14 days growth.

In table 7 the results of ten attempts to grow fragments of tissue are presented. The tissues used were from ten different patients. All attempts resulted in growth of fibroblasts which could be maintained for four weeks, at which time degeneration of the cells was observed. Two cultures from two patients were subcultured (M.F. and L. J.). In the former the subculture was observed to grow for three weeks and in the latter the subculture survived for two weeks. It is doubtful if such cultures would be of value, providing continuous cell lines were maintained, as only fibroblasts were observed in the cultures after the first 24-48 hours of growth. Since verruca is primarily a tumor of epithelial cells, it would seem reasonable that epithelial cells would be the most susceptible host for the agent.

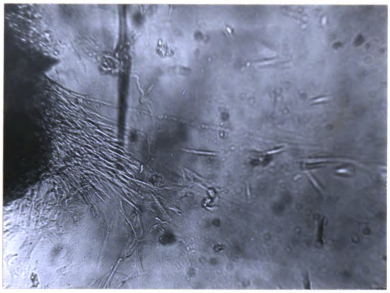


Figure 1. Implant of wart fragment (4 days, unstained, x200).

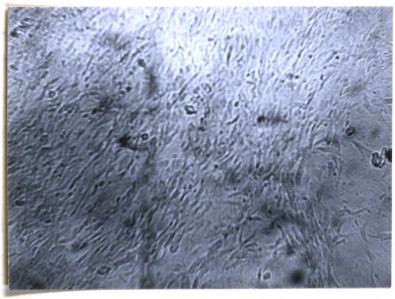


Figure 2. Fibroblast-like growth from wart implant (after 14 days, unstained, x200).

Table 7: Growth in primary cell cultures using fresh fragments of wart tissues.

Patient	Initial Culture Cells	Duration in Weeks	No. of Subcultures
C. P.	+ *	2	N.D.**
M. F.	+	2	3
C. H.	+	4	N.D.
K. L.	+	3	N.D.
P. F.	+	4	N.D.
J. M.	+	4	N.D.
T. J.	+	4	N.D.
L. J.	+	2	2
P. O.	+	4	N.D.
J. M.	+	4	N.D.

* + = Fibroblast growth.

** N.D. = Not done.

D. Cultivation of an agent in AU tissue cells

The history of the AU tissue cell culture has been described by Wheeler et al. (1957). The original cells came from a 17-year-old boy (AU) and were established as a cell line for the study of virus infection of the skin by Wheeler et al. The number of subcultures of this strain of cells was not known and no attempt has been made to record these data since the cell culture was received in this laboratory. The cells are epithelial and morphologically indistinguishable from the HeLa neoplastic cells of Gey (Gey et al., 1952). The nutrient requirement of the AU cells is complex but satisfactory growth takes place in a medium consisting of 80 per cent yeast extract medium with 20 per cent inactivated human serum as recommended by Wheeler. Later, it was determined that Eagle's basal medium could be substituted for the yeast extract medium. To maintain the cultures two per cent inactivated calf serum was used. Both growth and maintenance media contained antibiotics.

The AU cell line appears to be difficult to maintain as compared to well established cell lines. The human serum used has a great deal to do with the rate of growth as well as the length of time cultures can be maintained without spontaneous degeneration of the cell cultures. Serum from some individuals was found to destroy the cells.

The first attempts to cultivate the wart agent in AU cells were made with fresh wart tissues. Patients with multiple wart infections were chosen to provide ample wart material. Cell-free extracts and fragments (1 mm^3) from each patient were prepared and each was seeded into three tubes of AU cell cultures. The cultures were incubated at 37 C in the horizontal, stationary position and observed each 24 hours for morphological changes in the cells. Uninoculated cell cultures were observed as controls.

In those cultures receiving cell-free extracts of wart tissues, no changes were observed. Those cultures receiving fragments of wart tissues were found to be altered and, depending upon the amount of inoculum used, the cells in some of the cultures were detached from the tube wall. Although sterility control tests did not indicate the presence of bacteria, it was at first considered that the tissue cell cultures were contaminated. The procedure was repeated. This time fewer fragments of wart tissue per tube were used as inoculum and the cultures were observed several times during the first 48 hours of incubation. Again the AU cultures receiving cell-free inocula were indistinguishable from the cells in the control cultures. The cultures receiving wart tissue fragments were seen to change from their normal morphology to a rounded cell which aggregated and finally became separated from the cell sheet and tube wall. This cytopathic effect (CPE) is illustrated in figures 3, 4, and 5, and the normal cell sheet of the AU cells is shown in figure 6. Cultures showing cytopathic effect were subcultured in two ways. After the majority of the cells had become detached from the tube wall, the nutrient fluid and cells from each tube were pooled per specimen. The fluid was placed in centrifuge tubes and spun at slow speed in a horizontal centrifuge. The cell-free supernatant fluid was separated from the cells. Only enough fluid was left on the sedimented cells to act as a vehicle. The cell-free supernatant fluid was used as inoculum for fresh cultures of AU cells. The cell suspension (0.1 ml of heavy suspension) was also inoculated into fresh cultures. Both sets of cultures were incubated at 37 C and observed repeatedly. As in the original cultures, the cell-free inoculum produced no visible signs of cytopathic effect. Although the time between inoculation and effect was increased by several days, those cultures receiving the cellular

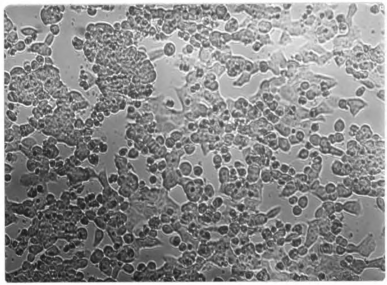


Figure 3. Early stages of cytopathic effect of AU cells seeded with fragments of wart tissue. (Unstained, x240).

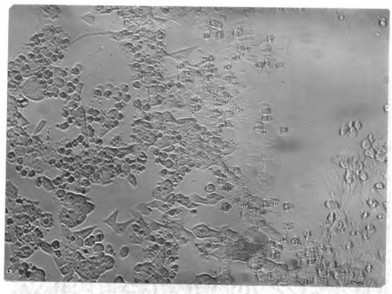


Figure 4. Cytopathic effect on AU cells. (Unstained, x240).

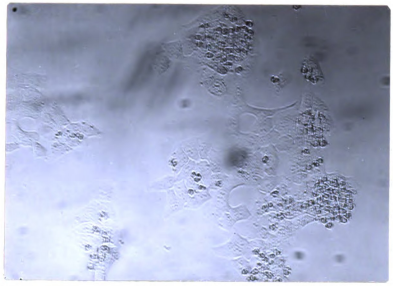


Figure 5. Cytopathic effect, end results.
(Unstained, x240).

Figure 6. Normal AU cells monolayers.
(Unstained, x240).

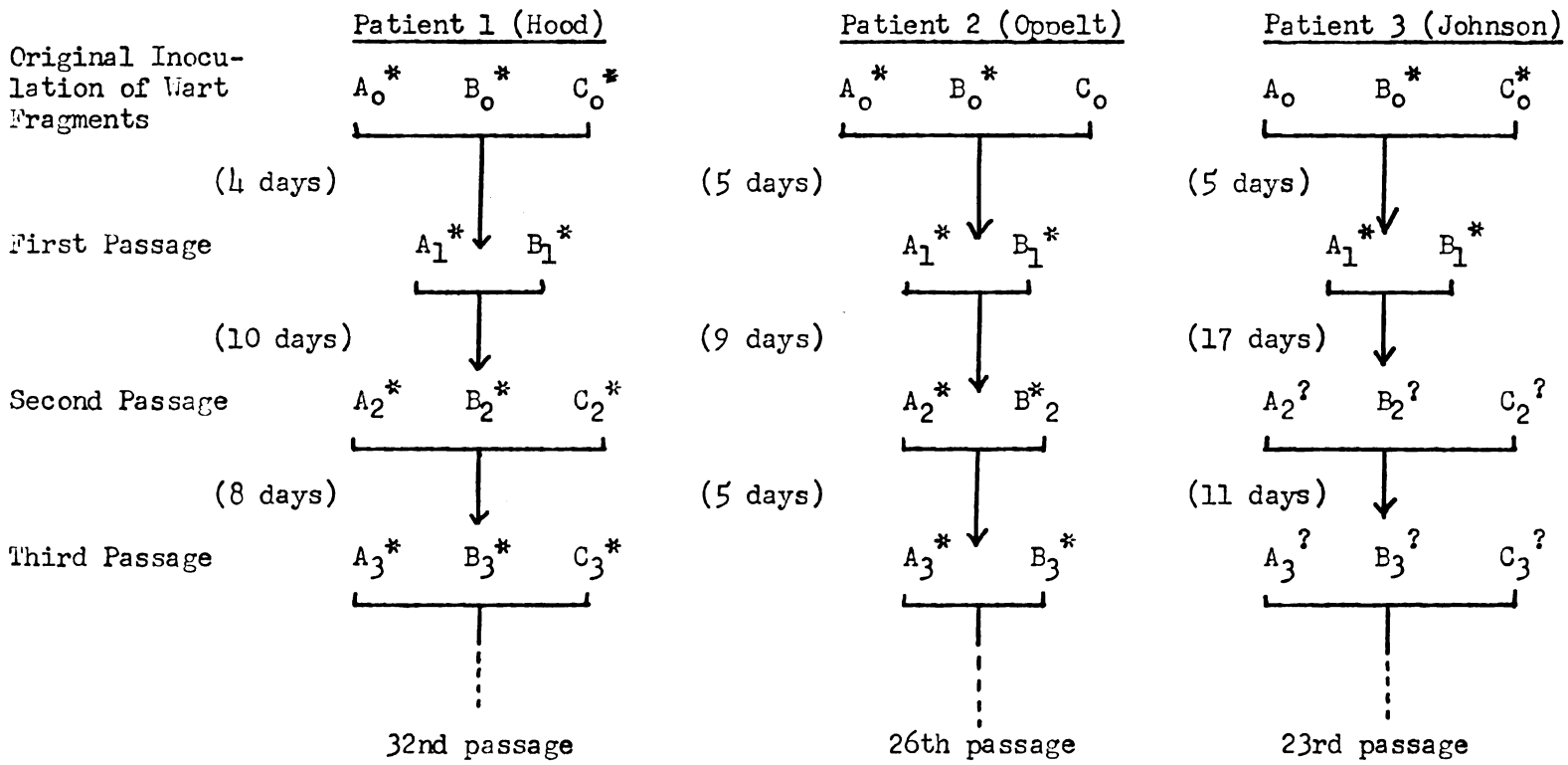
inocula again were found to exhibit cytopathic effect and the cells were detached from the wall of the tubes. Although the cultures receiving cell-free inocula showed no effect and were indistinguishable from the control cells, "blind" passages were made using the cell-free nutrient fluids as inocula. No evidence of cytopathic effect was seen in these series of cultures.

In table 8 typical results are given of the initial and secondary passages of wart-infected cellular inoculum on the AU cell cultures. Patient 1 (Hood), age 26, had a history of repeated wart infection on the skin of both hands. Patient 2 (Oppelt), age 28, also had had warts removed repeatedly. Patient 3 (Johnson), age 10, had extensive wart infections on the skin beneath the distal margin of thumbs and index fingers. The latter patient's infection was so extensive that the removal of additional tissues would produce disfiguration. The patient was considered extremely susceptible to infection by the wart agent.

To make certain that the wart agent was multiplying in the AU cell cultures, repeated serial passages were made using 0.1 ml of a heavy suspension of infected cells as inoculum. In table 8 the data are presented for three strains of the wart agent that have been serially passed 32, 26, and 23 times from patients, Hood, Oppelt, and Johnson respectively. The nutrient fluids were changed every second day in the serial passages and, considering the dilution and number of passages, there was little doubt that the agent was propagated from infected to uninfected cells.

In an attempt to understand the mechanism involved in this unusual circumstance, experiments were designed to disprove the results. Normal fragments of skin (1 mm^3) from parents of the patients from whom warts

Table 8: Initial and subcultures of wart agent from three patients in AU cell cultures.



* Tubes of tissue cell culture showing cytopathic effect.

** A_o .... B_1 ..... tubes inoculated in each passage.

were removed, normal skin from patients from whom warts had been removed, foreskin from newborn and normal skin from laboratory personnel were used as inoculum on AU cell cultures. Cytopathic effect was not observed when any of the normal skin tissue was used as inoculum. Normal-appearing, uninoculated AU cells were removed from the tube wall by physical means and seeded onto monolayers of AU cell cultures. These cultures were incubated and observed similarly to infected cultures but no cytopathic effects were produced in the cell cultures. To eliminate the possibility that wart tissues contained antibiotic resistant microorganisms not present in normal skin, repeated cultures were made of the inoculum-cell material to special culture media for bacteria. Special consideration was given and tests were made for the pleuropneumonia-like organisms. No cultivatable bacteria were recovered from these control tests. As will be seen later on, infected cells from serial passages were injected intracerebrally into animals without producing meningitis, a good test for the presence of bacteria.

The AU cells appeared to be the only cells showing evidence of infection from contact with wart tissue. To determine if other epithelial cell cultures could be infected with infected AU cells, six epithelial cell lines were tested. Table 9 represents the epithelial cell lines used. The AU cells used as inocula were subcultures of the agent using the patient's name to identify the strain. The infected AU cell cultures were divided into cell suspensions and cell-free nutrient fluids. Both samples were then used to inoculate cultures of the indicated (table 9) epithelial cell line.

Although different strains were used and different passages of the same strain, there was no evidence that the agent could be visually

Table 9: Attempts to cultivate the wart agent in other epithelial cells after isolation in the AU cell cultures.

AU Subculture Passage	Cell Line Cultures	Inocula	Observation Period in Days
Hood 1, 13, 14, 15* Oppelt 10, 12 Johnson 8, 10, 12 Anderson 1 Dallman 1 Sussex 5 Crail 2	Human embryonic skin (MAF)	Cell-free nutrient	14-21
Hood 1, 13, 14, 15 Oppelt 10, 12 Johnson 8, 10, 12 Anderson 1 Dallman 1 Sussex 5 Crail 2	Human embryonic skin (MAF)	Cell suspension	14-21
Hood 15, 18 Oppelt 12, 14 Johnson 12, 13	Monkey kidney	Cell-free nutrient	21
Hood 15, 18 Oppelt 12, 14 Johnson 12, 13	Monkey kidney	Cell suspension	21
Hood 13 Oppelt 10 Johnson 10	Human kidney and reticulosarcoma of lung (T-1)	Cell-free nutrient	21
Hood 13 Oppelt 10 Johnson 10	Human kidney and reticulosarcoma of lung (T-1)	Cell suspension	21
Hood 28 Oppelt 21 Johnson 21	HeLa	Cell-free nutrient	14
Hood 28 Oppelt 21 Johnson 21	HeLa	Cell suspension	14
Hood 20 Oppelt 16 Johnson 15	Embryonic chick liver	Cell-free nutrient	14
Hood 20 Oppelt 16 Johnson 15	Embryonic chick liver	Cell suspension	14
Hunter, original Anderson, original Porter, original	Human amnion (primary culture)	Cell suspension	21
Johnson, original Larkins, original	Human amnion (primary culture)	Cell-free nutrient	21

* Number following strain indicates passage number.

identified in any of the epithelial cell lines used. Control evidence of continued growth of infected AU cells is not presented in table 9. but, in all cases, the subsequent passage of the agent was maintained in AU cell cultures.

E. Mixed culture of wart and AU cells

To visualize what was happening when wart cells came in contact with normal AU cells, small fragments (1 mm^3) of fresh wart tissue were attached to the tube wall by the plasma clot method. After attachment of the implants, nutrient fluid was added to the tube and the culture was incubated horizontally at 37 C for 14 days. Observation of the implants showed that fibroblasts were growing out from the edge of the implants as illustrated in figures 1 and 2.

Normal AU cells, after removal from the tube wall by trypsin, were suspended in nutrient fluid at 100,000 cells per ml concentration. One ml of the normal AU cell suspension was then placed into each tube containing wart tissue implants. The AU cells were permitted to attach to the walls of the tube by horizontal incubation at 37 C. The tubes were placed so that the wart implants and AU cells were in the same plane. After 24 hours incubation the cultures were observed repeatedly to determine the results.

At the end of 24 hours incubation period, a zone of cytopathic effect was seen around the wart tissue implants. The zone consisting of rounded cells, continued to increase. By the fourth and fifth days the AU cells and wart tissue implants had degenerated and the cells were detached from the tube walls. Figures 7 and 8 show the zone of cytopathic effect.

The results verify the fact that the wart agent present in the implants was capable of infecting the AU cells and producing a progressive

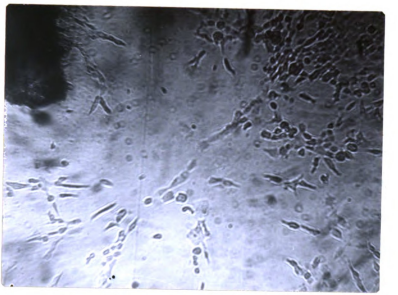


Figure 7. Cytopathic changes of wart tissue implant and AU cell culture (Unstained, x200).

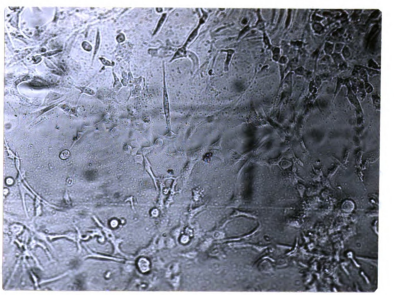


Figure 8. Zone of cytopathic change between wart tissue implant below, and AU cells above (Unstained, x200).

cytopathic effect. As the reaction started at the periphery of the implant and spread outward, it was considered that the agent in the wart implant diffused outwardly from infected to normal cell. The results observed were identical to plaque formation as seen in monolayers of cells infected with other viruses. In this case, the agent was present in wart tissue cells and not detectable in the fluid phase as is customary in plaque formation with other viruses. The infection was progressive as in the growth of virus forming a plaque. From the experiment it could not be determined whether the fibroblasts seen in the cultures were capable of infecting the AU cells or not. Although the predominant cells growing out from the implant were fibroblasts, occasional epithelial cells were present. The final result was removal of all cells from the wall of the tube.

F. Survey of agent in wart tissues from infected patients

When it was found that an agent that infected AU cell cultures was present in wart tissue, the next question to be answered was whether this agent could be repeatedly isolated from wart tissues.

Warts from patients were removed in the laboratory and the tissues were permitted to stand at 4 C in balanced salt solution containing antibiotics for 24 hours. Following the standing period, small fragments of tissue (1 mm^3) were removed from the wart tissue. Three tubes of AU monolayer cell cultures were seeded with tissues from each patient. The cultures were incubated at 37 C and observed daily for cellular changes. No attempts were made to subculture those cultures showing typical cytopathic effect. A decision as to whether the agent had been isolated was difficult to determine. For want of more information an isolation was considered when two or more of three tubes from the same specimen,

showed a marked cytopathic effect. Using this arbitrary criterion for an isolation the data were compiled in table 10. From 21 patients (30.43%) the agent was recovered. The average age of these patients was 22 years. In 48 patients (69.57%) the agent could not be recovered, yet the negative results came from a younger average age group than did the positive samples. These data gave results just the opposite of what we expected and will be discussed later. It must be noted that only 69 patients made up the total number of individuals tested.

G. Attempts to use animal serum replacing human serum for the growth of AU cell cultures

Proof that the agent isolated was the causative agent of warts depended upon whether it would produce verruca when introduced into or onto the skin of man. As the AU cell line required human serum for its growth and, as human sera may contain the jaundice virus, attempts were made to adapt the AU cells to a medium containing animal serum. It was also suspected that human serum might contain antibodies to the wart agent. If the latter were true, this might explain why virus was not found in the fluid phase of the cultures. Animal sera were available while human sera required additional time and arrangements.

Monolayer sheets of AU cells were prepared in standard tissue culture tubes using yeast extract medium plus 20 per cent human serum as nutrient. The cells were trypsinized by the standard method and placed in tubes for attachment and the growth phase. The nutrient fluid was yeast extract medium: only the serum concentration and origin was altered. The serum composition of the first passage was 75 per cent human serum and 25 per cent calf or horse serum (see table 11). All tubes were incubated at 37 C. in a horizontal position. When the cells

Table 10: Survey of agent present in wart tissues from 69 infected individuals

No. Patients	Results	Average age of Individuals (in years)	Location of Tissue				
			Hand	Foot	Arm	Leg	Unknown
21	Positive	22	23	1	1		
48	Negative	19	38	1	2	6	4
Total: 69			61	2	3	6	4

Table 11: Attempts to grow AU cells in medium containing animal sera

Type Serum	No. Attempts	Subculture Growth					
		1st	2nd	3rd	4th	5th	6th
Calf	3	+++	+++	+++	++	+	-
Calf	1	+++	++	+	-		
Calf	2	+++	+	-			
Horse	2	+++	++	-			
Human (control)	1	+++	+++	+++	+++	+++	+++

* +++ Excellent, ++ Good, + Fair, - No growth.

had formed a monolayer sheet, the cells were subcultured. The second passage nutrient fluid contained half human and half calf or horse serum. The subcultures were continued until the cells were grown in nutrient fluid containing no human serum.

The adaptive procedure above was followed by subculturing the cell line in the type of serum to which it had been adapted, i.e. calf or horse sera. In table 11 the results of these experiments show that only a single additional passage could be made when other than human serum was used in the nutrient fluids for the cells. By the second or third subculturing the cells failed to multiply in the provided nutrient. Although a total of eight attempts were made with sera from three calves and a horse, the results showed that human serum was an essential component in the nutrient of the AU cell cultures.

H. The effect of temperature of incubation upon bound and fluid-phase verruca agent

Kilham (1959) reported that when fibroma and myxoma viruses were grown in tissue cultures at 38 C the fluid phase did not contain virus. Only cells could be used to transfer the virus to subcultures. When the cultures were incubated at 36 C both the cells and fluid phase contained virus.

To determine if this were the case with the verruca agent, cultures of AU and monkey kidney cells were incubated at 35 and 37 C. To one set of three AU and three monkey kidney cell cultures, 0.1 ml of cell-free infected wart nutrient fluid was added to each tube. To a second set of three AU and three monkey kidney cell cultures, 0.1 ml of a heavy suspension of infected AU cells in nutrient fluid was added. This combination (table 12) was again repeated so that one set of three tubes

Table 12: The effect of 35 or 37 C incubation temperature on the ability of cells or cell-free passage material to infect AU and monkey kidney cell cultures

Strain and Passage No.	Inocula	Temp. of Incubation (C)	Cytopathic changes AU cells	Cytopathic changes Monkey kidney Cells	Days Observed
Hood 18	Cell-free	35	- *	-	12
	Cells	35	+ **	-	7-12
	Cell-free	37	-	-	12
	Cells	37	+	-	7-12
Oppelt 16	Cell-free	35	-	-	12
	Cells	35	+	-	7-12
	Cell-free	37	-	-	12
	Cells	37	+	-	7-12
Johnson 16	Cell-free	35	-	-	12
	Cells	35	+	-	7-12
	Cell-free	37	-	-	12
	Cells	37	+	+	7-12

* - No cytopathic effect observed.

** + Cytopathic effect observed.

each of AU and monkey kidney cells, infected with cells or cell-free material, from each of three lines of the agent could be incubated at 35 or 37 C respectively. The result of this experiment is given in table 12. There was no difference, regarding infectivity, whether the AU cells or monkey kidney cell cultures were incubated at 35 or 37 C. The cell-free inocula failed to produce cytopathic effect in both cell lines. Only when infected AU cells were inoculated onto AU cells was cytopathic effect observed. It would appear that the verruca agent was closely associated with the infected cell and unlike the fibroma or myxoma viruses, the few degrees difference in temperature of incubation did not release the agent from the cell to produce visible changes in those cultures receiving fluid phase inocula.

II. Tissue cell culture neutralization tests

Although neutralization tests using cells have not given complete success, some information can be derived from these tests. Tests described here were done in an attempt to learn more about the mechanism of infection from cell to cell rather than in a strict neutralization of the agent.

In early tests, gamma globulin was used as the neutralizing serum. Blank and Rake (1955) and our own experience indicated that warts usually regress at maturity or shortly thereafter. Reasoning that gamma globulin would contain neutralizing antibodies to warts, dilutions of gamma globulin were placed in contact with infected cells. The AU cells used were the Hood strain which had been serially subcultured. The technique used was the addition of dilutions of serum to heavy suspensions of infected AU cells suspended in nutrient fluid. No attempt was made to count the number of infected cells or determine an infective dose. Serum dilutions

were also made in nutrient fluid i.e., yeast extract medium. The gamma globulin used represented a 16 times concentration of pooled adult sera. The Hood sera were obtained by several bleedings of the same patient from which the wart tissue had originally been removed. Antisera were also made by repeated injection of rabbits with AU cells, infected AU cells and infected cell-free nutrient fluid.

The results of the neutralization tests are presented in table 13. The contents of the table are averages of several different neutralization tests. As controls, normal cells were maintained in nutrient fluid plus two per cent calf serum; normal AU cells were placed in contact with a normal sheet of AU cells; infected AU cells were seeded onto normal AU cell sheets; infected AU cells were mixed with one part normal calf serum and one part nutrient fluid.

The tests on gamma globulin were arranged so that one volume of infected cells was mixed with one volume of gamma globulin which had been diluted to represent 1x, 8x, and undiluted or 16x concentration as compared to normal serum.

The tests on Hood's (homologous) serum were one volume of infected AU cells mixed with undiluted, 1:1 and 1:2 dilutions of the serum in nutrient fluid. All sera were inactivated at 56 C for $\frac{1}{2}$ hour prior to use.

All mixtures were incubated 24 hours at 4 C prior to placing 0.1 ml of each mixture of cells and serum on several tubes containing monolayers of normal AU cells. The cultures were observed daily and the cytopathic effect, if present, recorded.

Table 13 shows that, compared to the controls, gamma globulin prevented infection of the cells in 16x concentration. When gamma globulin

Table 13: Results of neutralization tests employing mixtures of infected AU cells and gamma globulin or homologous serum

Days Observed	Controls				Gamma Globulin			Hood Serum		
	Normal AU cell Control	Normal AU Cell on AU Cells	Infected AU Cell on AU Cells	Infected AU cells & 1:1 Calf Serum on AU Cells	Infected AU Cells 1x G.G.	Infected AU Cells 8x G.G.	Infected AU Cells 16x G.G.	Infected AU Cells and Undil.	Infected AU cells 1:1 Dil.	Infected AU Cells 1:2 Dil.
1	0/21*	0/2	1/12	0/9	0/6	0/6	0/3	0/3	0/9	0/3
2	0/21	0/2	7/12	4/9	0/6	0/6	0/3	0/3	0/9	0/3
3	0/21	0/2	7/12	8/9	3/6	0/6	0/3	0/3	2/9	0/3
4	0/21	0/2	10/12	9/9	3/6	0/6	0/3	0/3	2/9	0/3
5	1/21	0/2	12/12	9/9	3/6	1/6	0/3	0/3	5/9	1/3
6	1/21	2/2	12/12	9/9	3/6	5/6	0/3	0/3	5/9	1/3

* Cytopathic effect/ tubes inoculated.

was diluted to 8x and 1x concentration, the infection was delayed for one or two days. With the undiluted Hood serum there also was protection from infection. Like the gamma globulin, when less concentrated serum was used, less protection was afforded. The data with both sera emphasized the necessity of using large amounts of antibodies to inhibit infection for when the sera were diluted there was some, but not absolute, protection against infection. That protection was not afforded by components present in normal serum was emphasized by the rapidity with which infected cells and calf serum control cultures showed cytopathic changes. It would appear from the data presented that gamma globulin, as well as the serum samples from the patient from whom the wart was removed, contained antibody against the wart agent.

The results of the neutralization tests using rabbit prepared antisera are shown in table 14. The results of these tests were erratic and no information could be gained. They were included, however, to present data for discussion.

The nature of the neutralization tests employed requires some judgment as to their validity. The tests with gamma globulin and the homologous serum afforded some protection against the infection of normal cells with infected cells. The test can not, on the other hand, be used as conclusive evidence of specific antibody in gamma globulin nor the serum from the patient.

III. Attempts to infect laboratory animals with verruca tissue and wart agent recovered in tissue cell cultures.

Because of the danger involved in introducing foreign cells and sera into humans, animals were inoculated with infected tissues and tissue cell cultures. Although the AU cells originally came from normal healthy skin

Table 14: Results of neutralization tests employing mixtures of infected AU cells and rabbit anti-infected AU cells, anti-normal AU cells and anti-infected cell-free nutrient fluid

Days Observed	Infected Cells on Normal AU Cells (Control)	AU Infected Cells and								
		Anti-Infected Cells Serum			Anti-Normal Cells Serum			Anti-Cell-Free Nutrient Fluid Serum		
		1:1	1:2	1:4	1:1	1:2	1:4	1:1	1:2	1:4
1	1/3*	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3
2	3/3	0/3	3/3	0/3	2/3	2/3	0/3	0/3	3/3	0/3
3	3/3	0/3	3/3	1/3	3/3	3/3	3/3	3/3	3/3	3/3
4	3/3	3/3	3/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3
5	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
6	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3

* Number showing cytopathic effect/ No. inoculated.

(Wheeler et al., 1957), the cells had been cultivated in vitro for several years. Morphologically they were indistinguishable from the HeLa cells (Gey et al., 1952) that were originated from neoplastic tissue. In like manner, the injection of human serum may introduce the serum jaundice virus. Potentially both the AU cells and the sera used in the nutrients were capable of producing unwanted results if introduced into susceptible individuals. In an attempt to circumvent these hazards, yet appraise the results of the tissue cell cultures, specimens were injected into laboratory animals.

A. Monkeys

The animals used were Macaca cynomolgus and were in good health when inoculated. Just prior to inoculation the hair on the abdominal surface or head was removed. The animals received three types of inocula: 1) Pieces of wart tissue removed from patients. The tissues were placed into tissue culture fluid containing streptomycin and penicillin over night at 4 C before being used as inoculum. 2) Tissue culture cells were used, i.e. infected AU cells were harvested from cultures after detachment of the cells from the tube wall. The cells were separated from the nutrient fluid by centrifugation at low speed and enough supernatant fluid was permitted to remain on the packed cells so that they could be drawn into a hypodermic syringe. 3) Cell-free nutrient fluids from infected serial subcultures. The fluids usually were the supernatant fluids from (2) above.

X-ray "tanning" was done by restraining the monkey and irradiating a belt 75 x 110 cm across the abdominal area shielding the unexposed area. The animal received 6 doses of from 100 to 300 r. every second day until a total of 1000 r. had been applied.

Two animals were injected with cortisone acetate and verruca tissue or tissue culture material. Each animal received 25 mg cortisone intramuscularly five times each week.

Monkey 1 (male, immature). This animal received three specimens intracutaneously. Fresh verruca tissue smaller than 1 mm^3 was drawn into a syringe in 0.05 ml of basal salt solution. The fragment of tissue was deposited by forcing the tissue fragment through a large gauge needle into the skin. At the same time, 0.05 ml of a heavy suspension of infected AU cells suspended in nutrient fluid and 0.05 ml of infected cell-free nutrient were injected into the skin at two additional sites.

The observation period on this animal was 7 months. Within seven to 10 days after injection, the injection site of the tissue culture fluid and cells was healed and no evidence of wart growth was found. The site receiving the verruca tissues appeared as a blanched, raised area for two to three weeks. This was followed by complete absorption of the foreign tissue. The animal remained healthy during the observation period and developed no evidence of abnormal tissue growth during the period.

Monkey 2 (male, immature). Prior to inoculation the animal was "tanned" by X-ray. The tanned belt was then used as the site of injections for wart material. All injections were given intracutaneously. The animal received: 1. Two fragments (less than 1 mm^3) of verruca tissue from two different patients (Fisher and Bloomquist). 2. (Oppelt strain) 0.05 ml infected AU cell-free fluid, and 0.05 ml infected AU cell suspension (12th serial subculture). 3. (Montgomery strain) 0.05 ml infected AU cell-free fluid, and 0.05 ml infected AU cell suspension (3rd serial subculture). 4. (Hood strain) 0.05 ml infected AU cell-free

fluid, and 0.05 ml infected AU cell suspension (13th serial subculture).

The animal was observed over seven months. The "tanned" area soon scaled off, the sites of liquid inocula rapidly healed but the sites receiving tissue fragments were slower to return to healthy skin. At no time during the observation period were there any signs or symptoms of disease observed in this animal.

Monkey 3 (immature). The animal received several small fragments of fresh verruca tissue (less than 1 mm^3) intracerebrally. The needle was inserted 6 mm below the meninges into the left hemisphere and 0.5 ml of saline solution containing the tissue fragments were deposited into the brain. The tissue used was freshly removed from a patient, age 11 (Hallead), who recently had developed a wart on her finger.

This animal was carefully observed daily for four and one-half months for evidence of neurotropic involvement. None occurred.

Monkey 4 (male, immature). This animal received infected AU tissue culture materials (Hood strain). Pools were made of the 15th through the 26th serial subculture of this strain. Both infected cells and nutrient fluids were used. Approximately 140 ml of material was centrifuged in a horizontal position to separate the cells from the fluid. The cells were ground in a mortar with a pestle and the liquid phase was then used to dilute the cell paste. The mixture was again recentrifuged at slow speed to remove gross particles. The supernatant fluid from slow speed centrifugation was then ultracentrifuged at 42,040 r.p.m. (114,610x g) for one hour. Four tubes containing 35 ml each were ultracentrifuged and, after the cycle, the top 32 ml of fluid was removed from each tube. The remaining three ml in each tube was used to rub down the walls of the tube. There was no visible sediment. The contents from each of the

four tubes were pooled and placed into the ultracentrifuge. The second cycle was one hour at 42,040 r.p.m. (110,660x g). The top five ml of fluid was removed from the remianing tube and the walls of the tube were rubbed with a glass rod with the final 2 ml of fluid remaining in the tube. A small insoluble sediment was present in the suspension. This was removed by slow speed, horizontal centrifugation. The supernatant fluid was used to inoculate a monkey. Four injections each consisting of 0.05 ml of concentrated tissue culture material was injected intracutaneously into four different sites on the abdomen of monkey 4. The animal was observed for seven months. Within ten days the sites of inoculation healed and no evidence of infection has been observed.

Monkey 5 (male, immature). Two days prior to being used this animal received 25 mg of cortisone acetate intramuscularly. The cortisone injections have been continued five times a week and to date (May 10, 1961) the animal has received 2.15 grams total. Fresh verruca tissues, after being freed from bacteria with antibiotics, were placed intracutaneously by trocar. Three circular fragments of wart tissue 1 mm in diameter and 0.5 mm thick were forced into the skin of the abdomen with the trocar plunger. The resulting inoculation left small incisions which were closed by applying a small piece sterile absorbent cotton saturated with collodion. Introduction of the inocula produced three raised blanched blebs on the skin. Within 3 weeks the blebs had disappeared leaving only a small scar at the site of inoculations. This animal has been observed daily when receiving cortisone injections and no growths have appeared at the injection sites. This animal is still under observation.

Monkey 6 (male, immature). Two months(59 days) prior to being injected with verruca material this animal was given 25 mg of cortisone

acetate intramuscularly five times weekly. To date the animal has received 1.2 grams total and is still receiving the drug. Fifty nine days after the first injection of cortisone, the monkey received four site injections of a heavy suspension of infected AU cells in nutrient fluid. The specimens were original tissue culture passages of wart tissues from adult patients who have repeated wart infection. Two intracutaneous injections of 0.125 ml of cells into two sites were made for each specimen. Following injection one site for each of the two specimens was painted with one per cent (in acetone) 7, 12-dimethylbenz-alpha-anthracene (Eastman Organic Distillation Products Co., Rochester 3, New York). This cocarcinogen was applied over the injection site in a 10 mm circular skin area. A single uninjected equal area was also painted with the drug as a control. This animal healed rapidly at the site of injection and has been under observations for five days. Observations will be continued on this animal.

B. Suckling mice

Mice have been used almost exclusively in attempts to isolate the causative agents of neoplasms. The recovery of agents by inoculating these animals with human tissues has resulted in some skepticism regarding the origin of the isolated agent. An example of these agents is leukemia by Gross (1951). For this reason, although mice were used, the emphasis was not placed on the use of this animal species.

The mice used were virus-susceptible Swiss white. Litters were pooled with a common mother if necessary to have sufficient number of animals. All suckling mice were less than three days old when inoculated. The inoculum consisted of wart tissue recently removed from the patient. The tissues were either extracted or cut into fragments. A heavy suspension

of infected AU cells (Oppelt strain) in the eighth subculture was also used. In table 15 the number of mice, inoculum, route and observation period are presented. During the observation period no evidence of abnormal growth was observed on any of the animals and none died. At the completion of the experiment all mice were sacrificed and the internal organs were observed for signs of abnormalities. None was found.

C. Chickens

Fertile white-leghorn eggs were obtained from a commercial source and were incubated until hatched. One day after hatching, the chicks were inoculated with cells from subcultures of three strains of the agent. Table 16 presents the number of day-old chicks inoculated with the various infected AU cultures.

Of the 27 chicks inoculated, 14 died during the 60-day observation period. Those birds ill or dead were examined at autopsy for evidence of abnormal growth. Careful examination of the organs of each dead bird revealed no abnormal growth.

D. Hamsters

The adult hamsters used in these experiments were purchased from commercial sources. Every effort was made to prevent cannibalism of the inoculated animals but the majority of deaths were due to this cause. In table 17 the inocula used, the days of observations and the deaths recorded in adult animals are listed. There were six deaths in the 20 animals injected with infected AU cells and all were partially or completely consumed by cage mates. Examination of internal organs, when possible, revealed no gross lesions. The observation period on these experimental animals ranged from 78 to 109 days and no abnormal growths were observed.

Table 15: Results of inoculating mice with fresh verruca tissue extracts, fragments and tissue cell cultures

No. Mice	Wart Material	Patient	Route	Volume (ml)	Observation Period in Days	Results
5	Extract	Miller	I.C.*	0.03	180	Negative
5	Extract	Field	I.C.*	0.03	180	Negative
3	Fragments	Davis	I.C.	0.03	365	Negative
4	Cell culture	Oppelt	I.C.	0.03	365	Negative

* I.C. = intracerebral; I.C. = intracutaneous.

Table 16: Day-old chicks inoculated with AU infected cell subcultures

No. of Chicks	Route and Volume (ml)	Inoculum	Days Observed	No. Dead/ No. Inoculated
8	0.01 I.C.*	Cells (Hood) 14th passage	60	4/8
8	0.01 I.C.	Cells (Oppelt) 14th passage	60	5/8
11	0.01 I.C.	Cells (Anderson) original passage	60	5/11

* I.C. = intracerebral.

Table 17: Inoculation of adult hamsters with infected and non-infected AU cells.

No. hamsters	Route and Volume (ml)	Inoculum	Days Observed	No. Dead/ No. Inoculated
5	0.05 I.C.*	Infected AU cells Hoos, 17th passage	109	1/5
5	0.05 I.Q.*	Infected AU cells Hood, 17th passage	109	4/5
5 (controls)	None		109	3/5
2 (controls)	0.05 I.C.	Normal AU cells	109	0/2
2 (controls)	0.05 I.Q.	Normal AU cells	109	1/2
5	0.05 I.C.	Infected AU cells Hood, 20th passage	78	0/5
5	0.05 I.Q.	Infected AU cells Hood, 20th passage	78	1/5
6 (controls)	None		78	0/6
4 (controls)	0.05 I.C.	Normal AU cells	78	0/4
4 (controls)	0.05 I.Q.	Normal AU cells	78	0/4

Summary

Inoculation	No. Dead/ No. Inoculated	Per cent Dead
Infected AU cells	6/20	30.0
Normal AU cells	1/12	8.3
None	3/11	27.3

* I.C. = intracerebral, I.Q. = intracutaneous.

The results are summarized at the bottom of table 17. In non-inoculated control adult animals there were three deaths in the 11 animals. Among the animals inoculated with normal AU cells there was 1 death. Six of 20 animals died after injections with wart-infected AU cells. Thirty per cent of the animals receiving infected AU cells died as compared to 27 per cent of the animals receiving no injections. It would appear from the results that infected AU cells did not produce growth or death when introduced into the adult hamsters.

E. Suckling hamsters

The suckling hamsters used were laboratory bred from normal animals. Ten suckling hamsters, one to three days old, were injected intracutaneously with 0.05 ml of a heavy suspension of normal or infected AU cells from the 26th (Hood) passage. The animals were observed for 32 to 33 days without showing any evidence of abnormality. The details of this experiment are illustrated in table 18.

F. Rabbits

Half grown white rabbits were injected intracutaneously with normal and wart-infected AU cells. The abdomen was freed of hair with clippers and each animal received 0.05 ml of heavy suspension of cells. Table 19 presents the data for this experiment.

All animals remained well during the observation period and none developed lesions at the site of injections.

IV. Electron microscope observations of the particles seen in verruca tissues

Strauss et al. (1949, 1950) presented electron micrographs representing the agent causing warts. His crystalline, virus-like cluster of

Table 18: Inoculation of suckling hamsters with normal and infected AU cells

No. Hamsters	Route and Volume in ml	Inoculum	Days Observed	No. Dead/No. Inoculated
4	0.05 I.C.*	AU cells, Hood, 26th passage	33	0/4
2	0.05 I.C.	AU cells, Hood, 26th passage	32	0/2
2 (controls)	0.05 I.C.	Normal AU cells	33	0/2
2 (controls)	0.05 I.C.	Normal AU cells	32	0/2

* I.C. = intracutaneous.

Table 19: Results of injecting rabbits with normal AU cells and infected AU cells

Animal No.	Inoculum	Days Observed	Results
1	4 areas, ultracentrifuge concentrated cell-free infected nutrient fluid (Hood strain)	90	Negative
2	Infected AU cells, Hood, 8th passage	90	Negative
3	Normal AU cells	90	Negative

particles derived from human wart tissues measured 52 mu but when found independent ranged from 56 - 86 mu. The individual particles were spherical in shape. Recently, Siegel (1960) attempted to confirm the work of Strauss et al. Siegel found uniform particles in extracts from verruca tissue but found them to measure 16 mu in size.

By following the method described by Strauss et al., verruca tissues were extracted and limited observations were made on verruca tissue, infected and normal AU cells.

Electron microscope observations presented spherical particles in preparations from verruca tissue and infected AU cells as well as a great deal of debris. The size-range of these particles varied from 50 to 75 mu. The preparations showed particles that were definitely not uniform, nor were the particles of any one size in abundance. Preparations of control AU cells did not show particles of the size-range found in either verruca tissues or infected AU cells.

DISCUSSION

There is no doubt that warts are caused by a filterable agent as repeated experiments using human volunteers have demonstrated. Although the injection of volunteers has reproduced wart growth, one common finding in the experiments was the prolonged incubation period. Usually it required at least six months after the initial injection before visual evidence of wart growth was noticed. Another common finding in the human injection studies was that, after a short growth period, the experimentally produced wart regressed rapidly.

A comparison of the human injection studies with the results obtained in tissue cultures inoculated with wart material cannot be made. The hosts' cells are not all destroyed by any infectious agent because of the hosts' many defensive mechanisms. In in vitro cultures, cells do not have the benefit of this protection and infection is rapid and overwhelming. Virus activity may not always be visible as cytopathic effect. Virus may enter the cell producing a latent infection or at any time may become cytocidal. Why the agent was cytocidal, i.e. producing cytopathic effect, when wart tissue cells were placed in contact with AU cell cultures and not with other epithelial cell lines is not known. The AU cell line originally came from normal skin, but so did the HuS 3075 line of epithelial cells. The former required a relatively simple growth medium while the latter required a complex growth medium, and it is possible that the nutrient requirements of the two different cell lines resulted in difference in susceptibility.

The reaction observed when wart tissue cells were placed in contact with AU cell cultures was first thought to be due to cytotoxic substances

in wart tissues. Twenty to thirty subcultures of three strains (Hood, Johnson and Oppelt) eliminated the possibility that this was the cause of cell degeneration. Further evidence that the agent isolated was associated with wart tissues was obtained by isolating the agent from 22 (30.43 per cent) of 69 different individuals.

The agent isolated from wart is the first such agent derived from growths of man. It appears from the work reported here that cell to cell transfer of the agent occurs since the experiments designed were not able to show infection with cell-free material. With agents producing tumor growth in animals, cell-free fluids have been shown to be infectious. The pattern shown by the wart agent and that of lymphomatosis in chickens is not dissimilar. Injections of tumor cells into susceptible birds rapidly results in a tumor growth at the site of injection with metastases and ultimate death of the bird. When on the other hand, cell-free extracts of the tumor are injected, an incubation period of 100 to 300 days is required before evidence of disease appears. The tumors developing in the latter case are diffused and generalized instead of a tumor mass.

Many diseases suspected of being of viral etiology have presented difficulties in isolating the agent. This difficulty has been overcome in some cases by placing diseased cells from the patient directly in contact with tissue cell cultures. Rowe et al. (1958) in this manner was able to isolate the salivary gland virus from children. Subcultures of the agent required that infected cells be transferred as no evidence of infection in vitro was observed when cell-free inocula were used. The agent of cytomegalic inclusions isolated by Weller et al. (1957) also required cell transfer initially but eventually cell-free fluids were shown to be infectious to tissue cell cultures. Weller, Witton and

Bell (1958) isolated the viruses of varicella and herpes zoster in tissue cell culture and again infected cultured cells were required to subculture the agent.

Stewart and Irwin (1960) described several agents isolated from neoplastic tissues in monolayer cell cultures. Although the original starting material was cell-free, the agents isolated from 3 different individual neoplastic tissues required cells to transfer the agent. All of these references support the theory that virus exists in cells and can be transferred to daughter cells without total destruction of the cells in the cultures. This was best illustrated by Temin and Rubin (1959) with Rous sarcoma virus.

The isolation of disease-producing agents from several different types of diseases, hitherto unsuccessful, has been accomplished by using the infected cell as the source of the agent. It can be predicted that many more agents, especially those associated with tumors, will be isolated by utilizing the cell to cell transfer of the agent within the tissue cell culture system.

The attempt to determine the type of immune response in wart infection was inconclusive. Gamma globulin and homologous serum contained an antibody which prevented infected cells from infecting normal cultured cells. The exact mechanisms of this inhibition of infection probably can be explained by the results obtained by Russell (1961) in which complement fixing antibodies were found against the wart agent in the patients' sera. Under these circumstances, the wart agent and antibodies appeared in the same individual. Spontaneous regression of warts usually, but not always, takes place at puberty (Blank and Rake, 1954) but it has not been determined whether this is due to antibody formation or other

causes such as local tissue resistance.

Animals injected with wart cells, infected tissue culture cells and cell-free nutrient fluids failed to produce any evidence of infection. If the agent is intimately associated with the cell as was shown by the tissue cell cultures it is not surprising that infection was not established in the animals. With this close relation between cell and agent, the transfer of the cells to a foreign species would result in immediate rejection of the transplant of cells. Cell-free inoculum containing the agent would also find adsorption and certainly penetration of the agent into foreign cells impossible.

The electron microscope studies reported do nothing to settle the differences as reported by Strauss et al. (1949, 1950) and Siegel (1960). It is sufficient to state that particles were observed in extracts from wart tissue cells and infected AU cells but not in normal AU cell extracts. The significance of these particles to the infective agent can only be speculated.

ADDENDUM

In April, Mendelson and Kligman (1961) reported that they had successfully isolated the virus of warts in monkey kidney tissue cells. Inoculation of human volunteers with cell-free nutrient fluids from the cultures produced wart growth in the skin of the volunteers. This is the first successful report of isolating the wart agent using monkey kidney cell cultures although others (Siegel and Novy, 1955) have attempted isolations as described by Mendelson and Kligman without success.

SUMMARY

1. More than 200 samples of verrucae from 195 patients were tested in 13 different types of tissue cell cultures in an attempt to isolate the wart agent.

2. Only when small fragments of fresh verruca tissue were placed in contact with AU cell cultures was cytopathic effect observed.

3. By cell to cell transfer three strains of the verruca agent were carried through 23, 26, and 32 serial subcultures respectively. In addition, the agent was successfully isolated from 30 per cent of 69 verruca samples.

4. Attempts to establish new human skin and verruca cell culture were unsuccessful.

5. Human gamma globulin and homologous patient's sera, from a single patient, were found to inhibit cell to cell infection *in vitro* in a cell-serum neutralization test. Diluted patient's serum or gamma globulin failed to give protection to normal cells indicating that large amounts of antibody were necessary to prevent infection.

6. Monkeys, rabbits, hamsters, mice, and chickens were injected with verruca cells, infected AU cells, and cell-free material. None gave evidence of infection.

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