FLUORESCENT ANTIBODY AND ACRIDINE ORANGE STUDIES OF THE HUMAN WART VIRUS IN CELL CULTURES OF NORMAL HUMAN SKIN

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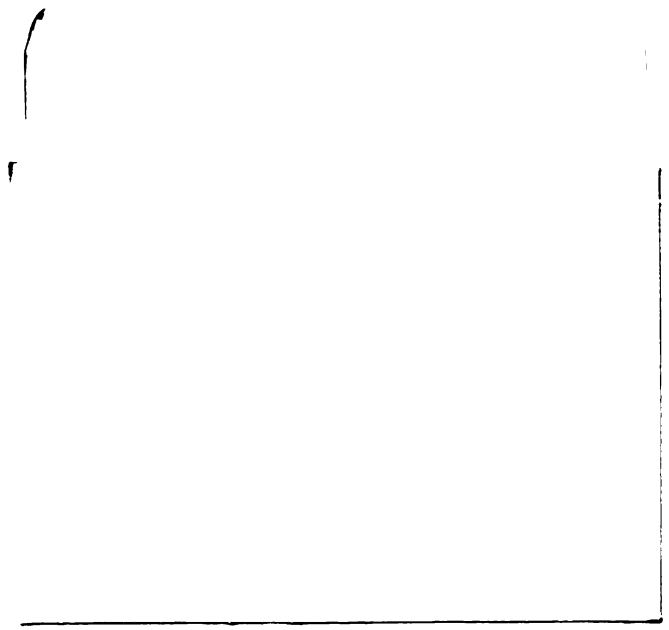

ABSTRACT

FLUORESCENT ANTIBODY AND ACRIDINE ORANGE STUDIES OF THE HUMAN WART VIRUS IN CELL CULTURES OF NORMAL HUMAN SKIN

by Josephine M. Magis

Acridine orange and fluorescent antibodies have been used in conjunction with fluorescence microscopy to ascertain whether or not the agent isolated in tissue cell cultures of normal human skin is the wart virus. Although serial propagation in vitro has been demonstrated by the cytopathic changes appearing in the subcultures, the virus failed to appear in the nutrient fluid phase (Hayashi, 1961). Thus it was necessary to locate the virus within the cell and determine the site of replication.

Through acridine orange staining of fixed coverslip cultures infected either by wart tissue from four different patients or by inoculation with cell suspensions from positive subcultures, it was found that within 24 to 48 hours infected cells contained intranuclear inclusions of deoxyribonucleic acid which resisted removal by deoxyribonuclease unless they were first digested with pepsin. At no time were these inclusions susceptible to ribonuclease digestion. These findings suggest that the deoxyribonucleic acid material might be the virus in its mature form covered with a protein coat.

In addition, virus-cell interactions were observed by immunochemical staining with fluorescein-tagged antibodies by the indirect method. Viral antigen was detected in the nucleus of infected cells in

acetone-fixed preparations within 48 hours post inoculation. The number of cells exhibiting this fluorescence increased with time and in some instances fluorescent particles were visible in the cytoplasm. Antigen could be detected if the inoculum consisted of freshly removed wart tissues, frozen wart tissues, warts stored in glycerin at 4° C., or cells from infected subcultures. Antisera both with antibody levels detected by complement fixation tests and with no such detectable antibodies exhibited positive fluorescent antibody reactions not demonstrated by sera from patients with no previous history of warts.

These results tend to support the thesis that the etiological agent of common warts, which has been isolated and propagated in vitro, is a DNA virus which replicates in the nucleus of the infected cell.

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By

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INTRODUCTION

One of the major areas of research today is in the field of human cancer. Although many of the tumors of animals have proved to be of viral etiology, it has not been sufficiently demonstrated to date that the causative agent of human cancers is a virus.

Since the turn of the century, it has been known that one of the benign tumors of man, verruca vulgaris or the common human wart, is caused by a virus. Thus far most of the investigations conducted have been studies of the virus in the wart tissue. Although these studies have not shown whether or not the virus is in some way stimulating the proliferation of the cells; nevertheless, the infected epidermal cells do present an interesting system for studying cell-virus relationships, first because they provide an example of a viral-induced tumor in man and second because cells which are stimulated to proliferate but in which the virus is not apparent can be easily identified, since they are found at precise anatomical sites in the wart tissue. This system might also yield vital information concerning the mechanism by which the virus is able to release the cells from their normal growth-restraining influences thereby leading to undue proliferation and tumor formation.

Although the wart virus has not been successfully transmitted to animals where studies could be performed under controlled conditions, there have been reports of the successful growth of the virus in tissue cell cultures. In this laboratory it has been found that the wart virus can be cultured in a cell line of normal human skin as demonstrated by its ability to produce a cytopathic effect in the infected cells (Hayashi, 1961). However, the virus can only be transferred

when intact infected cells are used as an inoculum as is the case with other viruses, i.e., herpes zoster (Rapp and Melnick, 1963). Since the wart virus could not be detected in the supernatant nutrient fluids of the infected cultures, it was decided to attempt to demonstrate the presence of the intracellular virus by the use of fluorescent dyes. If the virus could be discerned within the cell, this system could be utilized as a model in the study of the relationships existing between infected cells and tumor-producing viruses of human origin.

Two extremely versatile techniques have been developed in the past few years which have added much to our knowledge of the intracellular localization of viruses and of their developmental cycles. One is the fluorescent antibody method of Coons and Kaplan (1950) which locates specifically viral antigen. Since the wart virus has been shown by the complement fixation reaction (Russell, 1961) to stimulate antibody formation in certain individuals, these antisera could be utilized in the fluorescent antibody technique. The second is the acridine orange technique developed separately by Armstrong (1956) and von Bertalanffy and Bickis (1956) which can be employed for the detection of intracellular nucleic acids. When accompanied by nuclease digestion, it will locate specifically viral nucleic acids in the cell. In addition to being extremely sensitive and specific for viral antigen and nucleic acids respectively, both of these procedures are simple to perform and yield results which can be easily reproduced. Thus it may be assumed that these techniques, when applied to the cell cultures infected with the wart virus, will provide not only evidence of the presence of the agent in the cells but may also supply information regarding the developmental phases of the virus in the cell.

REVIEW OF LITERATURE

I. The Etiology of Warts

Common human warts are usually found on the hands and fingers but also occasionally on the forearms, knees, face, feet and scalp. They have been described pathologically and histologically by Ogilvie (1951) and Blank and Rake (1955). Warts are classified as papillomas or benign tumors of the skin and epithelial surfaces. They differ from malignant cancers in as much as they grow outward from the surface of origin, while cancers invariably grow downward. Macroscopically warts are extremely hard due to excessive keratinization, have a smooth margin, and may be surrounded by an erythematous inflammatory halo. Bunting et al. (1952) reported that these were the type of warts in which they most commonly found a virus. Microscopic examination of the tissue shows the growth to consist of a series of folds formed due to the elongation of the rete pegs, which project downward as finger-like processes showing a tendency to curve inward toward a central core. There is a corresponding elongation of the papillary processes which radiate from the central core. Sections also exhibit vacuolation of many cells in the prickle layer, shrivelling of the nuclei in the granular layer, and hyperplasia of the stratum corneum.

For many years people have known that warts can be spread by contact and will disappear in response to suggestion or even hypnosis, and thus many fascinating tales have evolved concerning their development and cure. The first recorded experiments demonstrating the infectious nature of warts were those of Jadassohn (1896) in which he injected ground up wart tissues intradermally into both himself and his

assistants. In 31 out of the 74 sites of inoculation he was able to reproduce warts. The viral nature of this infectious agent was again demonstrated in 1907 by Ciuffo (1907) who was able to produce warts on both his hands and those of the patients from whom the warts had originally been removed by the intradermal injection of Berkefeld filtrates of cornified flat warts. These results were confirmed by Serra (1908) and by Wile and Kingery (1919) who were able to induce warts by the introduction of cell-free filtrates. The production of a second generation wart was reported by Kingery (1921). By removing warts which had been previously produced experimentally, filtering the extract, and injecting this material into a volunteer, he was able to produce a wart. These procedures have been recently repeated by Goldschmidt and Kligman (1958) but they were unsuccessful when trying to produce warts with their extracts. However, Mendelson and Kligman (1961) reported the isolation and propagation of the wart virus in monkey kidney tissue cultures. After injecting the cell-free nutrient fluids of these infected cultures, they were able to produce wart growths in human volunteers. In all cases where warts have been produced by the introduction of infectious materials a period of 6 months or more elapsed between the time of injection and the appearance of the newly produced growth.

Few workers have been able to report the in vitro isolation and propagation of the virus of warts. Felsher (1947) was unsuccessful in his attempts to isolate the virus by the inoculation of the chick chorioallantoic membrane with material from cutaneous lesions. The first successful isolation was reported by Bivins (1953) who had removed with a curette, one of the 18 warts which had grown on his hand, made an extract of the material, and inoculated it onto the chorioallantoic membrane of 10-day old chick embryos. He was able to produce what he termed pearly white raised cysts at the sites of injection and was

able to transfer the agent using Berkefeld filtrates of the membranes. Unfortunately Siegel (1956) demonstrated that the isolate was not the virus of warts but a contaminating strain of avian pox virus. Cultivation in vitro was attempted by Siegel and Novy (1955) who ground up wart material and inoculated embryonating hen's eggs, HeLa cell cultures, and monkey kidney cells; but they were in no case able to detect the presence of a virus either by electron microscope studies or by its production of some type of pathological effect in the inoculated systems. In the same year that Mendelson and Kligman reported the isolation of the wart virus, Hayashi (1961) in investigations undertaken in this laboratory reported the isolation and serial propagation of the wart agent in cultures of normal human skin. This agent was neutralized by undiluted homologous patient's serum and by human gamma globulin.

One of the more fruitful lines of research in the studies of warts has been the investigation of the development of the virus in wart tissue using the electron microscope. Almeida et al. (1962) have found that the human wart virus is formed in association with the nucleoli of cells in the stratum spinosum. Virus particles gradually spread throughout the nuclei of the cells in the stratum granulosum and persisted as close-packed aggregates embedded in the substance of the stratum corneum. This correlated with the development of basophillic intranuclear inclusions which can be seen in the light microscope. They also found that the eosinophillic intranuclear inclusions described by Strauss et al. (1951) were due to keratin-like material being formed and were in no way related to the virus. The observations of Arwyn (1960), Blank et al. (1951), and Lipschütz (1924) agreed with these results. Arwyn also described a membrane surrounding the scattered virus particles which may be the former nuclear membrane. Bunting (1953) noted that the cells of the stratum corneum no longer contained nuclei but instead solid areas of regularly arranged closely packed particles

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of virus. In all of the investigations it has been ascertained that the virus is present in 50 percent or less of the warts examined.

Additional information concerning the developmental cycle of the virus of warts has resulted from the acridine orange studies of Williams (1961). Tissue sections of verruca vulgaris lesions exhibited Feulgen positive inclusion bodies in the upper rete layer and the horny layer which fluoresced yellow-green when stained with the fluorescent dye, acridine orange, indicative of the presence of deoxyribonucleic acid. After treatment with deoxyribonuclease, cells of normal skin did not retain the nuclear stain, while the intranuclear inclusions of the wart tissue remained stained demonstrating the presence of viral nucleic acid which is unaffected by the nuclease digestion (Furusawa and Cutting, 1961).

Observations with the electron microscope of the wart virus in tissue or of purified preparations have led to a controversy over the actual size of the wart virus. Strauss et al. (1949) removed human skin papillomas and prepared ultracentrifuged aqueous extracts of the material for the electron microscope by shadow casting with palladium alloy. Their measurements were 52 mμ for particles found in a crystalline-like array in the cell nucleus, while free particles averaged 68 mμ. Using thin sections of wart material Bunting (1953) measured the diameter as ranging between 20 to 38 mμ, which agrees with the data reported by Arwyn (1960) of an average 33 mμ. However, Siegel (1960) proposed 16 mμ as the average diameter of the sperical particles which he visualized when examining purified preparations sprayed onto collodin mounts. The discrepancies in these findings probably are a result of the different methods which were followed. Utilizing negative staining with phosphotungstic acid and shadow casting with palladium alloy, Williams et al. (1961) measured the diameter of the particles as 55 mμ. It was observed that the capsid of the virus was composed of 42

capsomeres arranged in 5:3:2 cubic symmetry (Horne and Wildy, 1961). These measurements presented evidence that the human wart virus is structurally similar to the polyoma virus (Wildy et al., 1961) which produces a wide spectrum of malignant tumors in several animals and the rabbit papilloma virus (Stone et al., 1959) which was visualized in the nuclei of cells from lesions which had been experimentally produced in cottontail rabbits. Williams' results were recently verified by Noyes (1964) with the examination in the electron microscope of purified fractions of the wart virus obtained by sucrose gradient centrifugation from over 100 warts. These purified particles measured 58 mμ in diameter and were positive for deoxyribonucleic acid when stained with acridine orange. In all studies of the virus in tissue sections cited here thus far, the virus has been found only in the nucleus of the cell and not in the cytoplasm. The presence of virus-like particles with an average diameter of 38 mμ in both the cytoplasm and nucleus of cells in ultrathin sections of human warts has been reported by Chapman et al. (1963). Although in some cases there was possibility of leakage of the viral particles through the nuclear membrane, further investigation led them to conclude that the clusters of viral particles were not due to artifacts.

Very little work has been done concerning the antigenicity of the human wart virus. Beard and Kidd (1936) could demonstrate no antigenic relationships among the papillomas of man, dogs, cattle, and rabbits by the neutralization tests. Antibodies to the virus have been detected by the neutralization test (Hayashi, 1961) and complement fixation reaction (Russell, 1961) in the sera of some patients from whom warts had been removed. When applying fluorescent antibody techniques to wart tissues, Epstein (1963) was unable to detect the presence of antigen. This may be explained by the fact that the quantity of antigen in the tissue is very small or perhaps that the virus may be in the early stages of development

and the antigenic protein coat has not been synthesized by the cell as yet. This was the explanation offered by Noyes and Mellors (1957) to interpret their findings that less papilloma virus antigen could be detected in the cells of infected domesticated rabbits than in those of wild rabbits. In order to study the reactions between divalent antibodies and univalent antigens, Almeida et al. (1963) immunized goats and rabbits with the human wart virus and polyoma virus. In electron micrographs antibody molecules could be seen linking the virus particles thus bringing about the formation of large aggregates.

II. Fluorescent Dyes and Their Utilization for the Study of Virus-Cell Relationships

The possible value of fluorescent microscopy in viral research was appreciated soon after the introduction of fluorescent dyes. With adequate activating light source and objectives of high numerical aperature, outstanding results could be obtained. The image observed with the fluorescence microscope is formed by the emission of light from the specimen itself. Thus detection of the object depends primarily upon the intensity of the light emitted. The presence of dispersed objects, the dimensions of which are close to or even beyond the limits of precise delineation by transmitted light, may be detected as a pattern of linear or point sources of light against a black background. Thus work was undertaken to study various fluorescent compounds and their possible applications.

Fluorescent Antibodies

By the preparation of R-salt-azo-benzidine-azo antityphoid and anticholera sera, Marrack (1934) first clearly demonstrated that prosthetic groups or dye molecules could be chemically linked to antibody

molecules without impairing the capacity of the antibody to react specifically with the homologous antigen. This finding formed the basis for the future investigations with fluorescent antibodies.

Coons et al. (1941) successfully conjugated beta anthrylisocyanate with antipneumococcal III serum. The conjugated serum retained its capacity to fix complement, to agglutinate type III pneumococci, and to passively sensitize guinea pigs to anaphylactic shock. When applied to the type III organism and exposed to ultraviolet light of wave length between 300 and 400 mμ, the dye fluoresced blue. However, since this blue fluorescence was the same as is normally emitted by tissues, the dye was not suitable for localizing pnuemococci in infected host cells. Therefore, Coons et al. (1942) substituted the green fluorescent dye, fluorescein isocyanate, which when used to stain tissue sections from infected mice enabled them to observe the distribution of the organism. Further studies showed that the reaction of dye with the antibody involved the formation of a carbamide linkage between the amino group of the protein and the isocyanate group of the fluorochrome (Coons and Kaplan, 1950). It was also found that the nonspecific uptake of the dye by the tissues could be significantly reduced by a number of absorptions of the conjugated antiserum with acetone dried tissue extracts (Coons et al., 1950).

Various other fluorescent dyes were then tested for their possible applications to this technique. Riggs et al. (1958) found that fluorescein isothiocyanate had all the desirable properties of the isocyanate compound but in addition was stable for periods of up to six months and emitted light of much higher intensity. A tetramethyl derivative of fluorescein, rhodamine, which fluoresced red and could be utilized as a second label for the simultaneous identification of two antigens, was synthesized by Silverstein (1957). Chadwick et al.(1958) prepared a sulfanyl chloride derivative of Lissamine Rhodamine RB 200

which also fluoresced red. If normal serum was used for conjugation with this dye and if the absorption procedures were eliminated, Smith et al. (1959) observed that this complex furnished an ideal nonspecific counterstain when used in conjunction with the fluorescein-tagged specific serum.

All of these dyes have been utilized in the direct fluorescent antibody method of staining described by Coons (1958) which consists of conjugating the specific antiserum of high titer with the dye and reacting this directly with the specimen to be examined. This has the major disadvantage that each serum to be applied must first be coupled with the dye. Thus Weller and Coons (1954) developed the indirect or layering method. This involves the formation of an initial antigen-antibody complex between the homologous antigen and in this case an unconjugated antiserum. When a specific conjugated antiglobulin is added to this initial complex, the antibody coated antigen combines with this conjugate and thereby becomes fluorescent. The method, therefore, offers the added advantage that a single conjugated antiglobin may be used for a number of unconjugated antisera; although, it has the disadvantage of increasing the nonspecific reactions and thus increasing the need for including many more controls in the test procedure.

These techniques have subsequently been utilized by many workers for the detection of viral antigens in infected cell and tissue systems. Among the first were Coons and co-workers (1950) who demonstrated virus and rickettsiae in tissues and were able to identify the rickettsiae of typhus and Rocky Mountain spotted fever in exudate smears and in frozen tissue sections. Fluorescein-labeled antibodies were utilized by Liu (1955) for the rapid diagnosis of human influenza infections from nasal smears. The agents isolated in tissue culture from measles patients were detected by Cohen et al. (1955) with the fluorescent antibody and complement fixation tests. Rapp et al. (1959) assayed the

number of infectious units in a suspension of measles virus by counting the number of fluorescent foci produced in infected coverslip tissue cultures. Direct and indirect immunofluorescence were applied by Riggs and Brown (1962) to the identification of enteroviruses and to the titration of antibodies in the sera of patients after infection or after vaccination.

Fluorescent antibodies have also lent themselves to the study of malignant tumors. In Rous Sarcoma, Malmgrem et al. (1960) and Vogt and Luykx (1963) found diffuse and spherical cytoplasmic fluorescent bodies, the amount of fluorescence demonstrated being directly related to the amount of tumor-producing virus injected into the tissue. The fluorescein-stained viral antigens of avian myeloblastosis were detected at the cell surface in chick embryo cultures by Vogt and Ruben (1960). Brown and Bittner (1961) have used the fluorescent antibody reaction as a method for assaying the potency of sera prepared against the mouse mammary tumor agent and have found good agreement with the results from serum neutralization tests.

In studies of the tumors induced by the polyoma virus, Sachs and Fogel (1960) found that the proportion of fluorescent cells increased in tumors that had been grown in vivo as cell transplants and that primary mouse tumors contained the highest percentage of fluorescent cells, primary rat and hamster tumors exhibiting only small amounts of fluorescence. Investigations into the developmental phases of the polyoma virus by Malmgrem et al. (1960a) and Fraser and Gharpure (1962) have shown that in early stages of infection viral antigen is localized mainly in the cytoplasm, while at 72 to 96 hours post infection fluorescent areas were present in the nuclei. In the papillomas of wild and domesticated rabbits, Noyes and Mellors (1957) found that Shope papilloma virus antigen was present only in the nucleus of the differentiating cells of the keratinized layers. Noyes (1959) reported that infected tissues of the

domesticated cottontail rabbits contained much less detectable fluorescence than those of the wild animals, the variation probably being due to the fact that the virus was in an inmature state in the domesticated animals.

Fluorescein-conjugated antisera have also been utilized to compare normal and malignant tissues. Louis (1958) observed that both spontaneous and experimental tumors of man completely lack the fluorescence found in the cytoplasm of the corresponding type of normal cells (antisera used being specific for the normal cells). Goldstein et al. (1959) compared cell tissue cultures of neoplastic origin and cells from freshly removed surgical specimens. They found that a common antigen existed in the cultured cells and cells from the same type of tumor from which they had originally been grown.

Lastly, it should be mentioned that the fluorescent antibody technique has also been applied to studies on cancer therapy. Viruses such as Egypt 101 (Noyes, 1955) and West Nile (Southam et al., 1958), which were known to be destructive to certain malignant cells, were injected into malignant tissues. The virus could be identified in the various cells, its effects upon the cells studied, and its therapeutic value determined.

Acridine Orange

Recently work with the fluorescent dyes has led to the discovery of the unusual polychromatic fluorescing properties of the compound 2,8-bis-dimethylaminoacridine, known as acridine orange. This basic fluorochrome was utilized by many of the original workers as a vital stain. The differential fluorescence of the various cell components or "Strugger effect" (Strugger, 1940) was thought to distinguish between the living and dead matter of the cell (fluorescing green and red respectively). More recent evidence indicates that these interpretations

are valid only in a limited range of circumstances. In studies of cells cultured with acridine orange, Wolf and Aronson (1961) found that as degeneration of the cells progressed they lost their red fluorescence while the nucleus was still intact and thus fluoresced green. If the criteria of the vitality test were applied, these cells, which were established to be irreversibly injured or dead, would have to be called living. These results established that the acridine orange stain can not be used as a self-sufficient vital stain but results can only be interpreted when additional information concerning the condition of the cells has been acquired. It was soon realized that this stain was much more valuable for the differentiation of nucleic acids.

Information concerning the reason for the variation of staining resulted from the observations of Schümmerfeder (1958). As the concentration of the dye increases in aqueous solutions, they exhibit a gradual change of fluorescent color from green to yellow to orange to red. This metachromasy (red fluorescence) is due to the formation of polymers by the cations of the fluorochrome in more concentrated solutions. The intermediate colors yellow and orange are due to different mixtures of the green fluorescent monomers and the red fluorescent polymers. The amount of dye taken up by fixed tissues from solution was found to increase with rising pH. With low concentrations of acridine orange (e.g., 0.01 per cent) the cytoplasm and nucleus stain quite differently when the pH of the dye solution is between 1.5 and 5.0. Within this range the nucleus no longer possesses the ability to take up the dye, thus remaining yellow-green. However, the cytoplasm continues to adsorb the dye molecules from solution thus becoming metachromatically stained. At high pH all the cellular components fluoresce red, whereas at very acid pH they fluoresce green.

The mechanisms of the cellular uptake of the fluorochrome have not yet been clearly elucidated, but it is evident that the major factor

involved is the affinity of the dye for the two types of nucleic acid, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). A number of theories have been formed to explain the results. Anderson et al. (1959) proposed that the interaction with the DNA-containing elements occurs instantaneously forming a stable complex resulting in yellow-green fluorescence, while the induction of red fluorescence in the RNA-containing structures is by comparison a slow process requiring several minutes.

A second theory was suggested by Steiner and Beers (1958). They believe two types of interactions occur between the acridine orange and nucleic acids. One type involves the bases and internucleotide phosphates of the polyribonucleotides, while the second reaction involves the terminal phosphates of both polyribonucleotides and DNA. Thus the nuclear DNA will bind only a small number of dye molecules and fluoresce green; while the RNA of the nucleolis and cytoplasm which contains a larger number of terminal phosphate groups and also include the reactive internucleotide phosphates and bases will bind a much larger number of dye molecules and consequently fluoresce red.

Another explanation has been recently advanced by Wolf and Aronson (1961). It depends upon the stacking of the acridine orange molecules onto the nucleic acid polymers. In living tissues the dye is bound to only a small number of isolated binding sites on the nucleic acid-protein complexes in the nucleus and cytoplasm, thus they fluoresce orthochromatically (green). With cellular injury macromolecular complexes in the cytoplasm of the cell are dissociated producing metachromasy. In a freshly killed cell, the nuclear DNA remains undenatured, thus little stacking occurs and it fluoresces green. Having been stripped of its protective protein, the RNA can now stack a large number of dye molecules and will fluoresce metachromatically. When cells are subjected to the standard fixation procedures of acridine orange staining

(Armstrong, 1956) which employ the acid fixative Carnoy's fluid, the bonds between nucleic acids and protein are broken and the nucleic acids are precipitated in a chemically altered form. Now both will stain metachromatically. This may be altered by staining at acid pH, which partially suppresses the binding of the dye, so that the fixed DNA will bind the fluorochrome only at isolated sites staining orthochromatically, while the RNA will stack and aggregate more dye and stain metachromatically. Until more information is available, it is difficult to say which of the theories is correct, but it is readily seen that they do agree that the concentration and binding of the acridine orange molecules play an important role in the reaction.

Additional evidence as to the specificity of the dye for the nucleic acids resulted from nuclease digestion tests. Armstrong (1959) reported that the green fluorescing materials are susceptible only to deoxyribonuclease (DNase), while the red fluorescing components are susceptible only to ribonuclease (RNase).

Carnoy-fixed smears of purified DNA viruses such as polyoma (Mayor, 1961a), SV-40 (Mayor et al., 1963), K-virus (Mattern and co-workers, 1963), Tipula iridescent and vaccinia (Armstrong and Niven, 1957) all stain brilliant yellow-green with acridine orange. This characteristic staining is susceptible to DNase upon prior digestion with a proteolytic enzyme such as pepsin but is not susceptible to RNase digestion. When staining bacteriophage T2 Anderson et al. (1959) observed the same type fluorescence; however, it was susceptible to pretreatment with only DNase suggesting that the protein coat affords less protection to bacterial viruses. Mayor and Hill (1961) observed that stained concentrates of the single stranded DNA phase ΦX-174 demonstrated flame red fluorescence which was susceptible to DNase but not RNase digestion. Thus it appears that single stranded DNA behaves metachromatically. This has been confirmed by Bradley

and Felsenfeld (1959). When heat denaturing native DNA, thereby destroying its two-stranded helical structure, they found there was an increase in the dye-dye interactions due to increased stacking of the polyanions, thus it stained metachromatically.

Similar investigations have been conducted with the RNA viruses. Poliovirus and tobacco mosaic virus (Mayor and Diwan, 1961) and influenza virus (Anderson et al. 1959) have been classified as RNA agents due to the brilliant red fluorescence which is susceptible to RNase digestion and is exhibited by the fixed purified smears.

Acridine orange staining had originally been utilized for the histochemical differentiation of cellular nucleic acids by von Bertlanffy and Bickis (1956) and Armstrong (1956). When Dart and Turner (1959) found that the dye could also be employed to detect increases in the DNA and RNA content of neoplastic cells, virologists immediately realized that this cytochemical stain might also yield valuable information regarding virus-cell relationships in infected systems.

Anderson et al. (1959) observed quantitative differences in the RNA- and DNA-containing structures of virus-infected tissues. Fluorochrome staining revealed distinctive chemical changes in the nuclei of the cells infected with adenovirus. The alterations coincided with virus propagation and maturation. These findings verified that this technique afforded colorimetric chemical differentiation of the virus during the process of infection.

With acridine orange stained preparations, observation of the cytochemical patterns of virus-host cell interaction has lead to the possibility of grouping viruses according to the type of nucleic acid which they contain and to their established sites of replication and maturation. Among the RNA-containing viruses, West Nile encephalitis (Anderson et al., 1959) and reovirus (Rhim et al., 1962 and Mayor, 1963) demonstrate only a cytoplasmic replication cycle, while the

Mengo virus (Franklin, 1962) and poliovirus (Mayor, 1961) exhibit both nuclear and cytoplasmic involvement. Anderson et al. (1959) observed that with influenza virus, another RNA virus, the final stages of the maturation process occurred at the cell periphery where broad bands of red fluorescence could be seen in the cytoplasm. In all cases there was increased RNA (red) fluorescence susceptible to prior RNase digestion when the mature virus was present within the cell.

Differentiation of the mature DNA viruses is possible as the particles are susceptible to DNase digestion after proteolytic enzyme treatment, while the cellular DNA is removed with only DNase digestion. Among those viruses which demonstrate only a nuclear replication cycle are the adenoviruses (Armstrong and Hopper, 1959) and polyoma virus (Williams and Sheinin, 1961). Acridine orange studies have revealed evidence of both nuclear and cytoplasmic phases for the DNA-containing viruses SV-40 (Mayor et al., 1962) and human cytomegalovirus (McAllister et al., 1963). Thus far only cytoplasmic sites of replication have been demonstrated for ectromelia virus in Erhlich ascites tumor cells (Furusawa and Cutting, 1960), vaccinia virus in infected HeLa cells (Loh and Riggs, 1961), and the virus of molluscum contagiosum in monkey kidney cells (Raskin, 1963).

Many of these acridine orange studies of viral nucleic acid replication have been accompanied by fluorescent antibody studies to detect the presence of newly synthesized viral antigen in the infected cells. These additional results confirm the presence of the virus in the cells and also supply information concerning the sites and processes of synthesis of the viral protein coat.

The virus of warts has been isolated in tissue culture; however, its presence thus far has only been detected serologically by neutralization and complement fixation tests. As so much vital information concerning the detection and replication of viruses in cells has been gained by the

application of fluorescent dyes, perhaps this approach might provide evidence that the agent isolated in tissue culture is the wart virus. Presented here are the results of such an effort.

MATERIALS AND METHODS

Growth of AU Cell Cultures

This study utilized the epithelial-like AU cell culture which was originated by Wheeler et al. (1957) from the normal skin cells of a 17 year-old boy (AU). The cell line has been serially propagated for a few years in this laboratory and recently has been adapted to grow in medium supplemented with inactivated fetal calf serum. These cells have previously been shown to be susceptible to the wart agent (Hayashi, 1961) as they show a characteristic cytopathic effect (CPE) when infected with the agent. All cells were grown at 37° C. on acid-cleaned glass surfaces in yeast extract medium (YEM, Robertson et al., 1955) which consists of Hanks' balanced salt solution (BSS, Hanks and Wallace, 1949) with 0.1 per cent yeast extract, 0.35 per cent glucose, 0.002 per cent phenol red as pH indicator, and antibiotics containing 100 micrograms of streptomycin and 100 units of penicillin per ml. The growth medium was supplemented with 20 per cent inactivated fetal calf serum, while maintenance medium was supplemented with 2 per cent inactivated fetal calf serum. The pH of all media was adjusted to 7-7.2 with 7 per cent $NaHCO_3$.

Uninfected cell cultures were detached from glass surfaces with 0.25 per cent trypsin (1:250, Difco) in calcium free Hanks' balanced salt solution. New cultures were seeded with 1×10^5 viable cells per ml as determined by counting a portion of the cells suspended in crystal violet in a hemocytometer and adjusting the remaining cells to the desired concentration in growth medium. As the wart agent can only be serially passed by the transfer of infected cells (Hayashi, 1961) and not by the

transfer of supernatant fluid, infected cell cultures exhibiting the characteristic CPE were serially transferred by separating the cells remaining attached to the surface of the culture tube with a rubber policeman and centrifuging the suspension at low speed for 5 minutes. The cells were resuspended in 0.2 ml. of nutrient medium and added to 48 hour cultures of AU cells.

Growth of Cells in Leighton Tubes

Leighton tubes containing 11 x 25 mm coverslips were seeded with one ml. of growth medium containing approximately 5×10^5 viable cells and were incubated at 37° C. Growth medium consisted of YEM supplemented with 20 per cent inactivated fetal calf serum and 0.5 per cent lactalbumin hydrolysate (Difco). Cultures were allowed to propagate for 48 to 72 hours or until a satisfactory confluent monolayer was obtained. Growth medium was then replaced by maintenance medium and cultures were inoculated with small fragments of wart tissue or with 0.2 ml. of a heavy suspension of infected cells from serial passages. Cultures were reincubated at 37° C. and samples were removed and fixed for staining with acridine orange or fluorescein isothiocyanate conjugated antibodies at intervals of 24, 48, 72, 96, and 120 hours post infection.

Sterility Tests

At two intervals during the period covered by this study the AU cell cultures were tested for pleuropneumonia-like organisms by seeding samples of the cells and growth media into special pleuropneumonia media (PPLO, Difco). In both cases after incubation for 4 to 5 days cultures were negative for the growth of these contaminants.

Wart Tissues

Wart specimens were removed from patients by a dermatologist in the laboratory by total enucleation (Ulbrick et al., 1957). Tissues were placed directly into Hanks' balanced salt solution containing antibiotics and held at 4°C. for 24 to 48 hours. They were then minced into very small fragments with the blade of a scalpel. Tissue fragments from each individual patient were pooled and 7 to 10 were inoculated into each cell culture.

Wart specimens which had been frozen at -20°C. or stored in glycerol at 4°C. for a period of at least 2 to 3 years were also utilized in the fluorescent antibody studies. These were ground with a mortar and pestle. The material was suspended in 5 ml. of growth media and inoculated into 48 hour cell cultures in Leighton tubes.

Cytochemical Techniques

Acridine orange

Fixation.--Coverslip cultures were removed from the Leighton tubes with a long wire hook and were immediately fixed for 5 minutes at room temperature in Carnoy's fluid, which consists of 60 per cent ethyl alcohol, 30 per cent chloroform, and 10 per cent acetic acid. Fixed preparations were rehydrated by brief rinses in 80 per cent, 70 per cent, and 50 per cent ethanol and in distilled water.

Staining Procedures

The procedure followed was that described by Mayor (1961) and Mayor and Diwan (1961). This can be outlined as follows:

1. Fixed coverslip preparations were rinsed in two changes of McIlvaine's citric acid-disodium phosphate buffer at pH 4 for 2 minutes.

2. Cultures were stained for 8 to 10 minutes at pH 4 in McIlvaine's buffer containing 0.01 per cent acridine orange.
3. Stained coverslips were washed in two changes of citrate-phosphate buffer for three minutes.
4. Washed preparations were mounted on standard microscope slides using buffer at pH 4 as the mounting media.
5. Mounts were examined with fluorescent microscopy.

Reagents:

A stock solution of 0.1 per cent acridine orange was prepared in distilled water and diluted 1:10 with McIlvaine's buffer prior to use. The buffer solution was prepared by adding 12.29 ml. of 0.1 M citric acid to 7.71 ml. of 0.2 M disodium phosphate. These solutions will remain stable when refrigerated at 4°C. for approximately 1 week (Dart and Turner, 1959).

Digestion Tests

If cells had previously been stained with acridine orange, they were destained prior to digestion by rinsing in 50 per cent ethyl alcohol for 3 to 5 minutes as described by Furusawa and Cutting (1960).

DNase digestion

Fixed preparations were digested by incubation at 37° C. for 1 hour with 0.01 per cent deoxyribonuclease 2 x crystallized (Nutritional Biochemicals Corporation) in McIlvaine's buffer pH 6.6 (prepared by adding 5.45 ml. 0.1 M citric acid to 14.55 ml. of 0.2 M disodium phosphate) containing 0.1 per cent $MgSO_4$.

RNase digestion

Preparations were digested by incubation for 1 hour at 37° C. with 0.05 per cent ribonuclease 5 x crystallized (Nutritional Biochemicals Corporation) at pH 7 in glass-distilled water.

Pepsin digestion

Preparations were treated with 0.02 per cent pepsin Granular N. F. 1:3000 (Pfanstiehl Laboratories, Inc.) in 0.02 N HCl for 10 minutes at 37° C.

All enzyme treated preparations were immediately rinsed and brought to pH 4 in McIlvaine's buffer. Staining with acridine orange was achieved as previously described.

Controls consisted of preparations incubated under similar conditions with McIlvaine's buffer or distilled water instead of enzyme.

Fluorescent Antibody Studies

Antisera from patients

Some of the sera utilized had been taken from patients from whom warts were removed, and it had been stored at -20° C. Two of these sera (JP and DA) were known to exhibit a low level of complement fixing antibodies against a wart antigen, while others tested were known to be negative (Russell, 1961). Normal serum was collected from the author who had no prior history of warts. All of the sera were inactivated at 56° C. for 10 minutes prior to use. In some of the experiments the sera utilized were first adsorbed with heavy suspensions of normal AU cells by incubation overnight at 4° C. in order to assure the specificity of the antigen-antibody reaction. It was found that the amount of non-specific fluorescence was reduced.

Antiglobin conjugate

Rabbit antihuman globulin labeled with fluorescein isothiocyanate was obtained from Microbiological Associates, Inc., Bethesda, Md. Lissamine Rhodamine RB-200 (Smith et al., 1959) conjugated with bovine albumin was added to the reconstituted fluorescein-conjugated antiserum globulin for better visualization of the sites of antigen deposits

in the cells as this dye is taken up in a non-specific manner by the background cellular substances. This mixture was stored at 4°C. until utilized.

Fixation of cells

Coverslip cultures of AU cells, either uninfected or infected, were removed from the Leighton tubes with a long wire hook and air dried for 30 minutes. Cells were then fixed in cold acetone (ethanol destroys the antigenicity of viruses, Coons, 1958) for 5 minutes and again air dried for 30 minutes. These preparations were either stained immediately or stored at -20°C. until stained.

Staining procedure

The wart antigen was detected in infected AU cell cultures by the indirect method first described by Weller and Coons (1954). This consists of the following steps:

1. Specific serum diluted 1:10 (to help eliminate non-specific reactions) was overlayed on the acetone-fixed preparations for 30 minutes in a humid atmosphere (a petri dish containing moist cotton) at room temperature or overnight at 4°C.
2. The coverslips were washed with mild agitation with 3 changes of phosphate buffered saline solution (0.01 M. pH 7.5) for 30 minutes.
3. The specimen was stained by overlaying with the Rhodamine-fluorescein conjugated antihuman rabbit globulin for 30 minutes at room temperature.
4. Coverslips were rinsed by mild agitation in 3 changes of either carbonate-bicarbonate buffer (0.5 M, pH 9.0, Pital and Janowitz, 1963) or phosphate buffered saline solution at pH 7.5 for approximately 30 minutes.

5. Coverslips were mounted in buffered glycerol on standard microscope slides.

6. Mounts were examined with fluorescent microscopy.

Reagents:

Carbonate-bicarbonate buffer, pH 9.0

Solution A
Na_2CO_3 . . . . . 5.30 grams
H_2O . . . . . to make 100 ml.

Solution B
$NaHCO_3$. . . . . 4.20 grams
H_2O . . . . . to make 100 ml.

Add 4.4 ml. of solution A to 100 ml. of solution B and check pH. Add more solution A if pH 9 has not been attained.

Phosphate Buffered Saline, pH 7.5

Solution A
Na_2HPO_4 . . . . . 1.40 grams
H_2O . . . . . to make 100 ml.

Add 84.10 ml. of solution A to 15.90 ml. of solution B from above. Add 8.50 grams of NaCl in a liter container. Dilute to one liter with water.

Controls employed were as follows:

1. Infected cells without specific antiserum + conjugate
2. Infected cells with specific antiserum - conjugate
3. Infected cells with normal serum + conjugate
4. Uninfected cells with specific antiserum + conjugate
5. Uninfected cells with specific antiserum - conjugate
6. Uninfected cells without specific antiserum + conjugate.

Fluorescent Microscopy and Photography

A Carl Zeiss microscope equipped with a super pressure mercury lamp Osram 200 was used with appropriate exciter and barrier filter

systems for observation. Filter systems were varied as follows: (1) for general UV light excitation, exciter filter I (BG12) and exciter filter III (UG2) whereby fluorescent light 350-500 mμ can be observed-+- blue barrier filter (OG4 or OG5) which transmit light above 500 mμ was used for fluorescent antibody studies, (2) exciter filter I (BG12) and exciter filter II (BG12) for intense BV excitation and observation of fluorescence light in range of 350-500 mμ -+- blue barrier filter (OG4 or OG5) transmitting light above 500 mμ was used for fluorescent antibody studies, (3) exciter filter III (UG2) for intense UV excitation and observation of fluorescent light of 330-400 mμ -+- red barrier filter (BG23) which transmits light above 350 mμ was used for acridine orange-stained preparations.

Mounts were viewed and the percentage of infected cells was determined by counting either fluorescent foci or DNA inclusions in 4 or 5 fields of cells under low power.

All photographs were taken with an Exakta camera using Kodak High Speed Ektachrome, Daylight Type. The time of exposure was varied between 30 seconds to 2 minutes for the acridine orange-stained preparations and between 2 and 5 minutes for the fluorescein-stained preparations. At least 3 to 4 pictures were necessary to insure proper exposure.

RESULTS

I. Growth of Wart Virus in AU Tissue Cell Monolayer at 37° C.

As found by Hayashi (1961) the cultures inoculated with fragments of freshly removed wart tissue exhibited a progressive cytopathic effect. Cells in the monolayer became either rounded or more angular and showed increased cytoplasmic granulation. As degeneration continued, the cells began to aggregate and detach from the wall of the tube usually by the 5th or 6th day post inoculation, although the time varied with specimens from different individuals. Uninoculated control cell cultures did not demonstrate this effect.

Heavy suspensions of infected cells from these cultures were used for the serial passage of the wart agent, since at no time could the presence of virus be detected in the supernatant nutrient fluids of the infected cultures by the serial passage of these fluids. The infected cell monolayers again demonstrated the cytopathic effect described above and degeneration of the sheet was usually evident by 8 to 10 days post inoculation. Varying numbers of passages of the viral-infected cultures were subsequently made and observed. The time required for degeneration of the cells fluctuated between 5 and 9 days. Uninfected cell cultures were also transferred as controls but at no time were the characteristic cytopathic effects in evidence. As adequate methods are not available, the amount of infectious virus being passed could not be determined.

Since media supplemented with lactalbumin hydrolysate was required to maintain the growth of coverslip cell cultures in Leighton

tubes, comparative studies were conducted to determine whether or not the additional nutrient material affected the susceptibility of the cell monolayers to infection with the wart agent. It was found that cultures grown in the supplemented media were also susceptible to the wart virus and exhibited a cytopathic effect, although an additional 1 to 3 days post inoculation elapsed before degeneration of the cell sheet became evident. An explanation for these findings may be that the large number of amino acids and other nutrients in the lactalbumin hydrolysate are utilized for the synthesis of increased amounts of essential materials in the cell making it more "healthy" and thus slowing down cellular degeneration upon infection.

II. Results of Acridine Orange Staining

The red fluorescent ribonucleic acid (RNA) of the cytoplasm and nucleolus and yellow-green deoxyribonucleic acid (DNA) fluorescing nucleus of normal uninfected cell tissue cultures can be seen in Figures 1, 2, 3, and 4. These prints are not true representations of what is observed in the fluorescent microscope. The yellow-green fluorescence of the nuclear and chromatin materials has been distorted to only a bright yellow fluorescence in many of the prints. This is probably due to the developing methods followed by the photo services as the transparencies showed no such distortions. Because of this distortion it is at times difficult to differentiate the fluorescence due to viral nucleic acid if nuclease digestion had not been performed.

The presence and replication of the wart virus in the cells from tissue cultures inoculated with freshly removed wart material were studied by fixing and staining preparations at 24, 48, 72, 96 and 120 hours post inoculation. Figure 5 shows cells from a culture 24 hours after inoculation with wart material from patient DI who had approximately

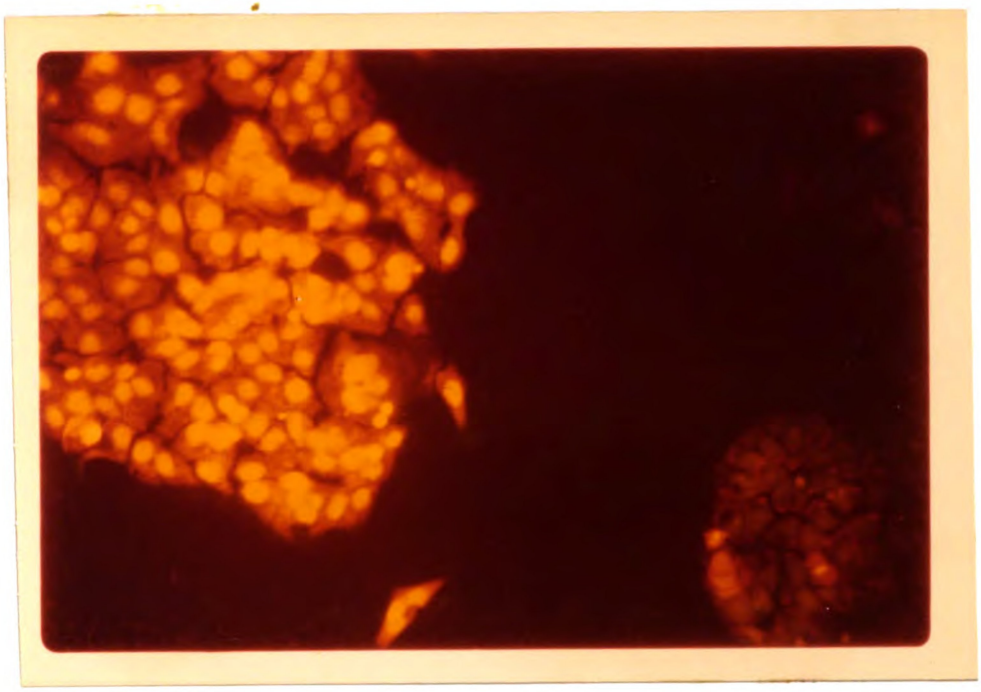

Figure 1. Normal AU cell culture stained with acridine orange after 24 hours on maintenance media. (x200)

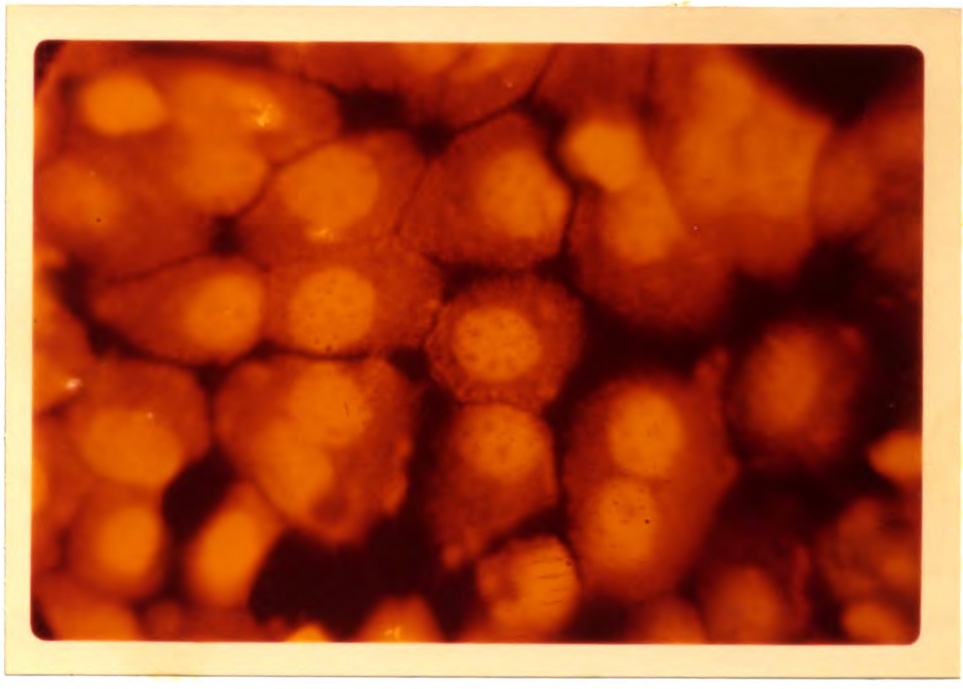

Figure 2. Normal AU cell culture stained with acridine orange after 48 hours on maintenance media. (x800)

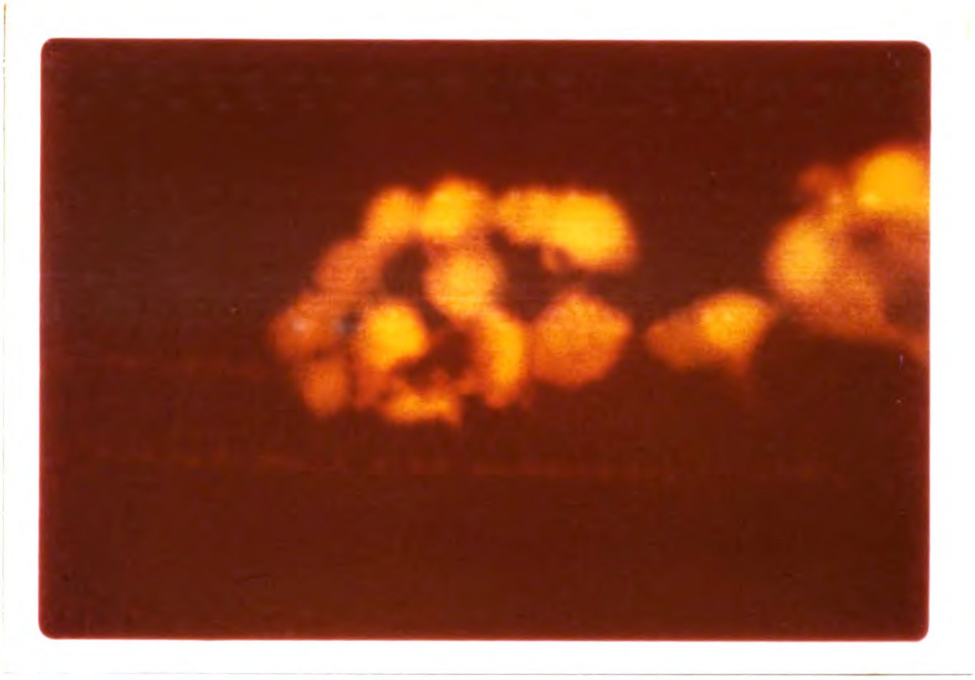

Figure 3. Normal AU cells stained with acridine orange after 72 hours on maintenance media. (x800)

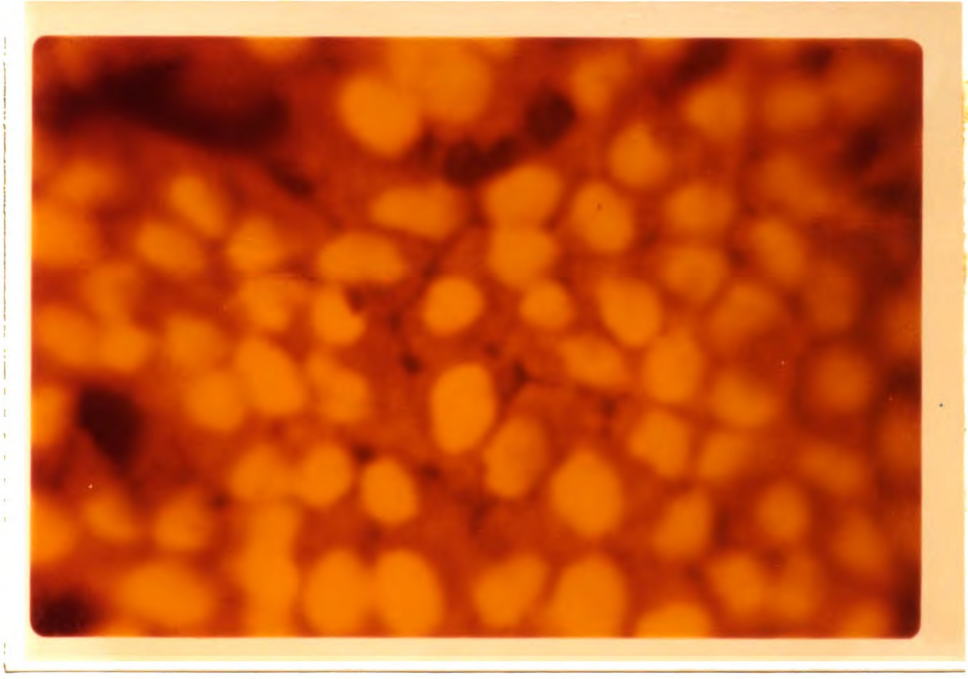

Figure 4. Normal AU cells stained with acridine orange after 96 hours on maintenance media. (x800)

17 warts removed from his hands at one time and within 3 months more had appeared. Since he seems to have no resistance to the virus, one would expect these tissues to contain virus in the infectious state. The cells in Figure 5 were beginning to degenerate as they had been grown without lactalbumin hydrolysate. The same type degeneration was seen in the controls, but the latter did not exhibit the increase in DNA and bright yellow fluorescence seen in the inoculated cultures. This was the only case when a culture inoculated with wart tissues demonstrated intranuclear DNA inclusions (white triangle), resistant to DNase digestion unless first treated with pepsin, at 24 hours post inoculation. With all other wart tissues, although cells did at times exhibit an increase in DNA fluorescing material, it was removed by DNase digestion, indicating the viral nucleic acid was not covered by a protein coat.

By 48 hours after inoculation, most of the infected cultures showed increased intranuclear fluorescence as seen in Figures 6 and 7. The nuclei in some of the cells appeared somewhat enlarged and contained fluorescent material which was not removed by DNase digestion as shown in Figure 8. Figure 9 illustrates an uninfected control which was also digested with DNase. The inclusions in Figure 8 were not removed by RNase digestion, but proved to be susceptible to DNase digestion after treatment with pepsin. The inclusions were present in only 1 to 2 per cent of the cells in the monolayer.

Subsequently, studies were made of the cell monolayers at 72, 96, and 120 hours post inoculation. Figures 10 and 11 demonstrate the appearance of the infected cells within 72 hours. Many of the cells were beginning to round up and were showing cytoplasmic degeneration, the nuclei becoming eccentric with brilliant and concentrated DNA staining and containing no visible nucleoli. As seen in Figure 11, the DNA in these fixed preparations was not susceptible to DNase digestion, but it

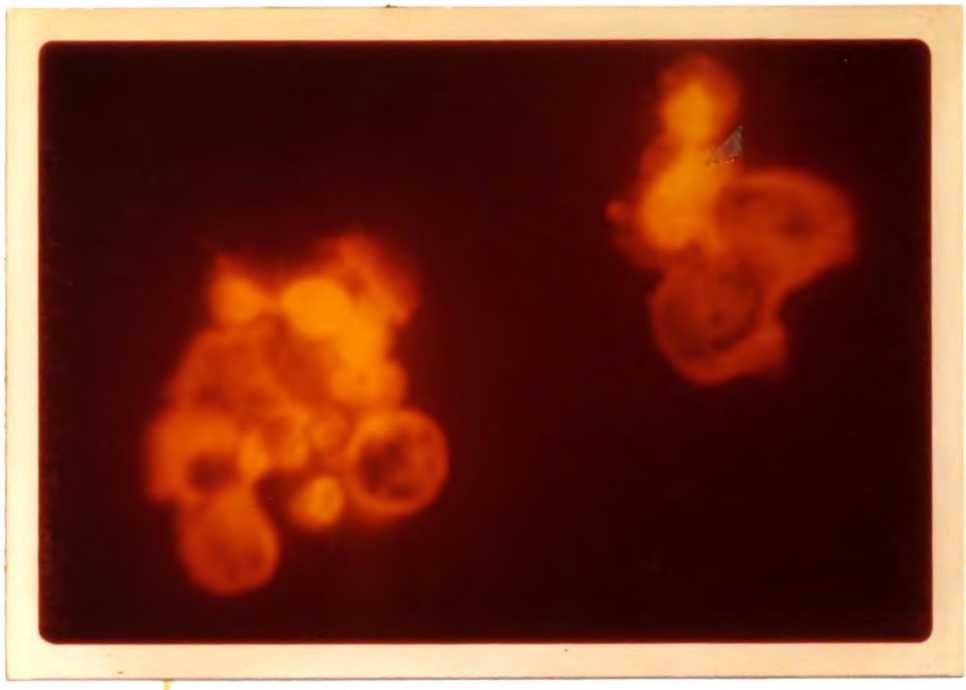

Figure 5. Acridine orange-stained AU cell culture 24 hours post inoculation with wart material from DI. (x800)

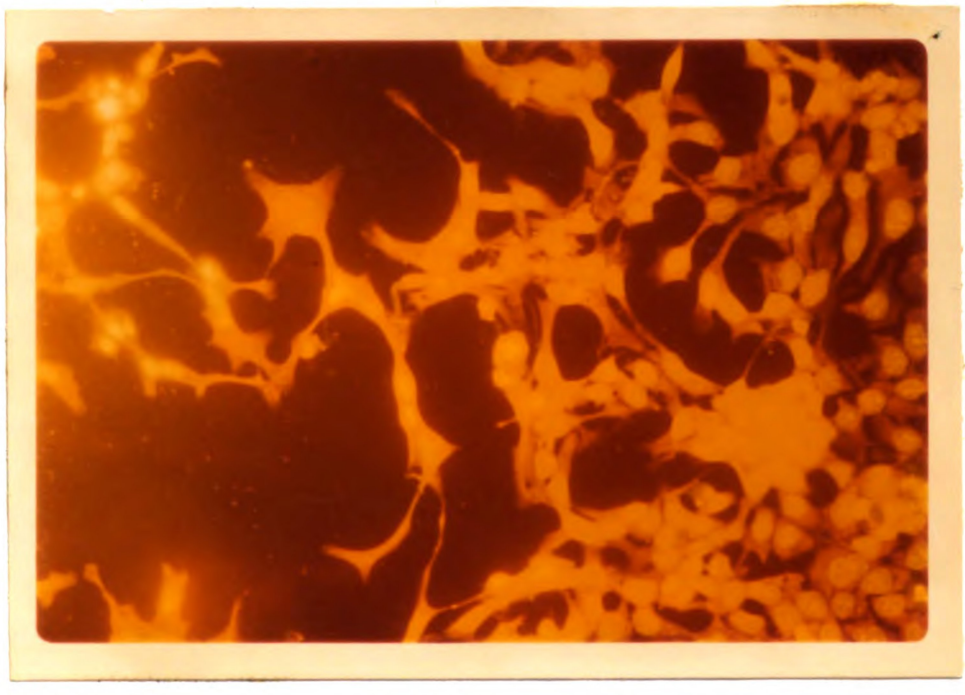

Figure 6. Acridine orange-stained AU cell culture 48 hours post inoculation with wart material from GB. (x200)

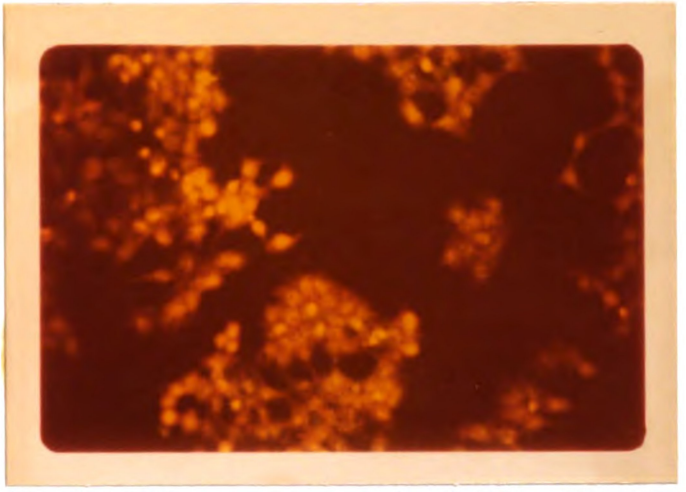

Figure 7. Acridine orange-stained AU cell culture 48 hours post inoculation with wart material from MEF. (x100)

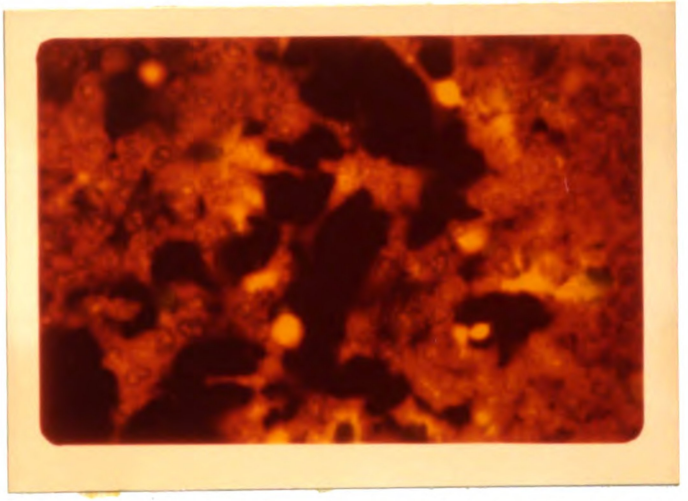

Figure 8. Acridine orange-stain of DNase digested AU cell culture 48 hours after inoculation with wart material from MEF. (x200)

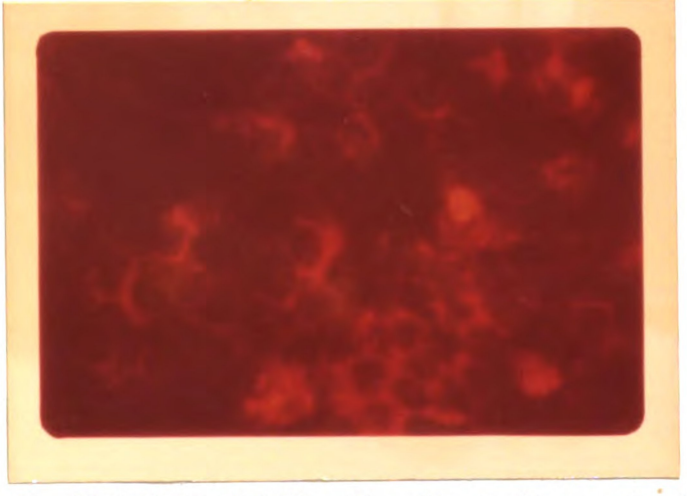

Figure 9. Uninfected AU cell culture digested with DNase and stained with acridine orange. (x400)

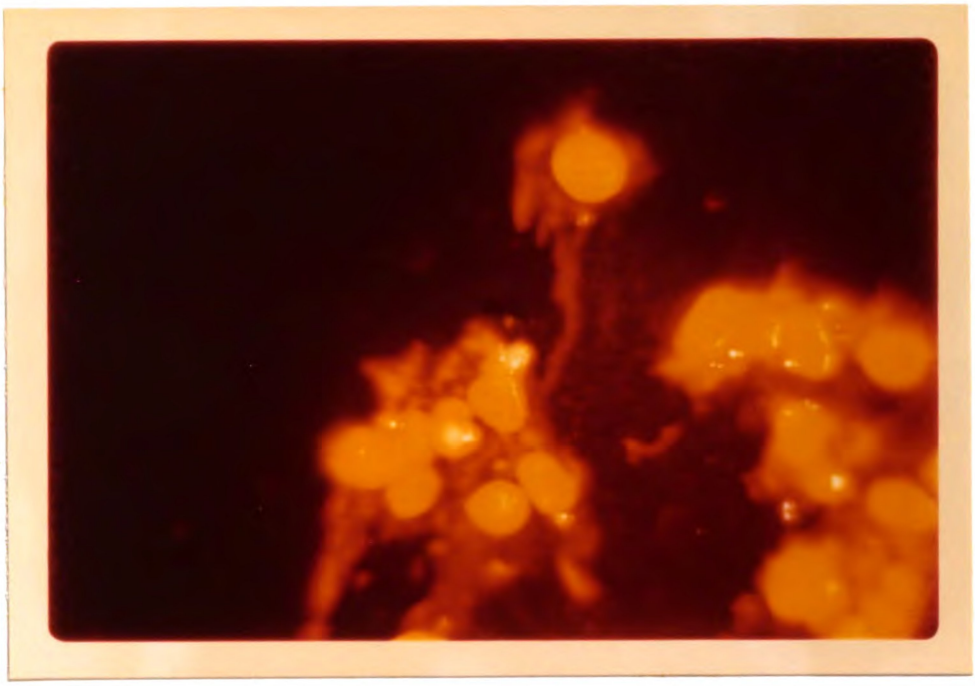

Figure 10. Acridine orange-stained AU cell culture 72 hours post inoculation with wart tissue from DI. (x800)

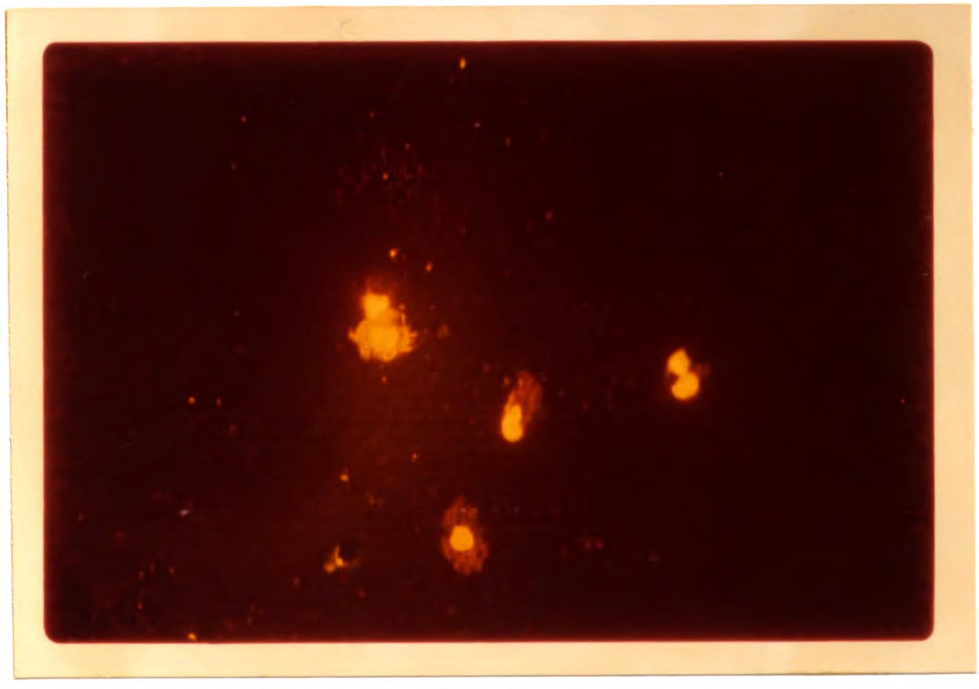

Figure 11. DNase digested and acridine orange-stained cell culture 72 hours post inoculation with wart tissue from DI. (x400)

could be removed if cells were first treated with pepsin and then subjected to DNase digestion. It was not susceptible to RNase digestion.

By 96 hours post inoculation, the cells presented an interesting aspect. As can be seen in Figure 12, upon DNase digestion, the DNA inclusions remained intact; however, the nucleus of one of the cells in the field appears to be disrupting and liberating small particles of DNA material into the cytoplasm. This cell is surrounded by some others in which the virus has not yet reached this stage of maturation. Thus only diffuse intranuclear DNA staining material, resistant to DNase digestion, is visible. This is similar to what is seen in Figure 13 in a preparation fixed 120 hours after inoculation and digested with DNase.

These results do not present an accurate picture of the patterns of viral synthesis in the infected cells, since the cultures were inoculated with wart tissues containing undetermined amounts of infectious virus. They do, however, demonstrate that the infected cells display similar patterns even when inoculated with wart tissues from different patients. Within 48 hours one to two per cent of the cells in the monolayer contained DNA staining material resistant to DNase digestion. As more time elapsed after inoculation, the number of cells containing intranuclear DNA inclusions increased until approximately ten to fifteen per cent of the monolayer seemed to be infected by the fifth day. The results of the studies conducted on cultures inoculated with tissues procured from different patients have been summarized in Table 1.

The findings of the acridine orange staining of monolayers inoculated with cell suspensions from subcultures of the wart agent isolated from patient DI can be seen in Figures 14 through 21 and are also summarized in Table 1. Some of the cells as seen in Figures 14, 16, and 19 contain large amounts of clumped intranuclear DNA fluorescence, the nuclei appearing somewhat shrunken and distorted. In other cases,

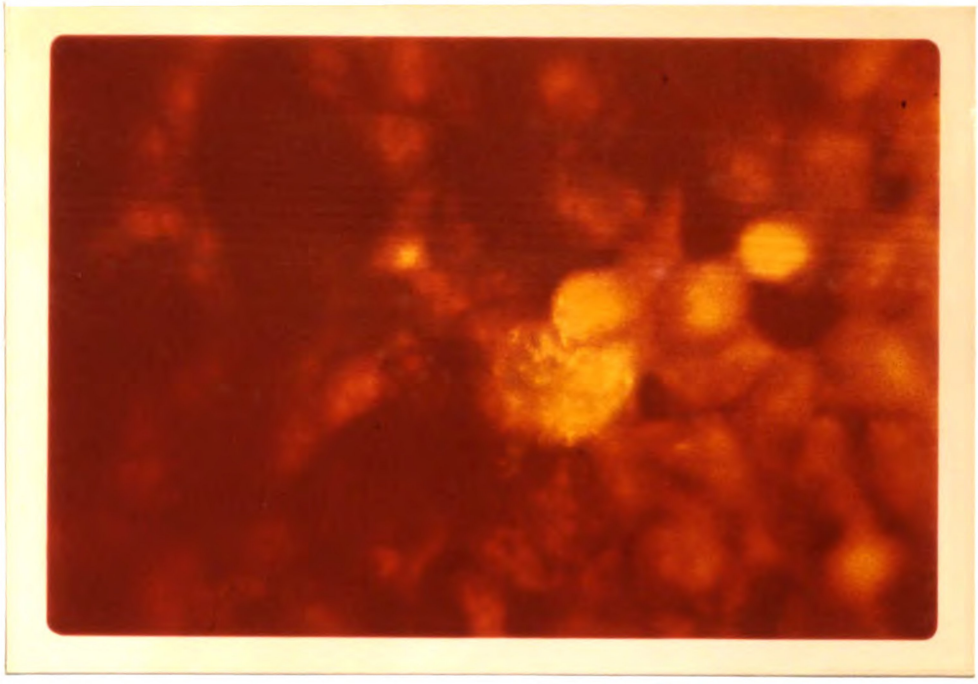

Figure 12. DNase digested and acridine orange-stained cell culture 96 hours post inoculation with wart specimen from SD. (x800)

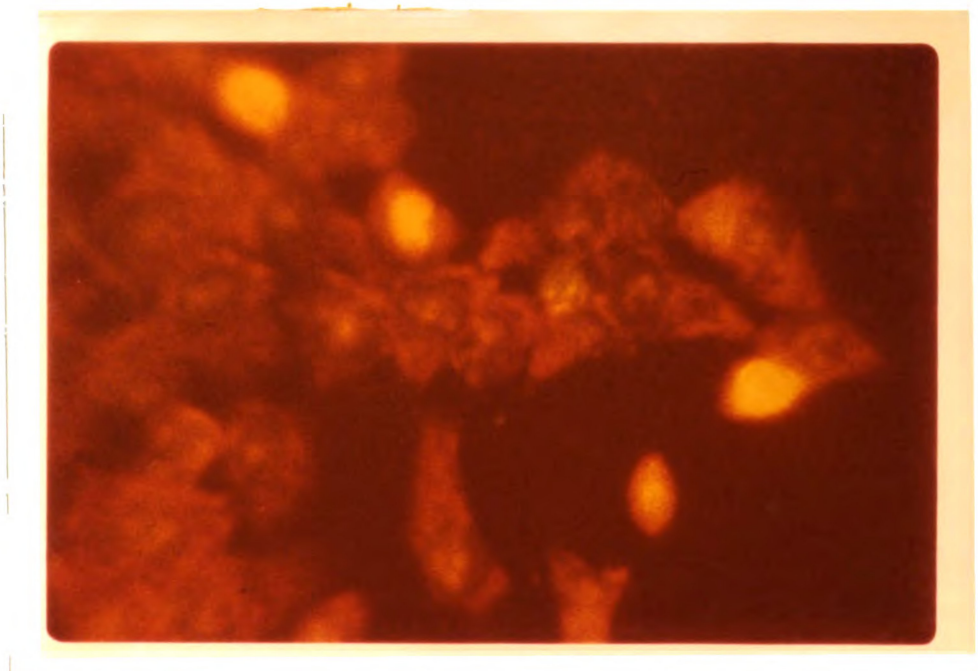

Figure 13. DNase digested and acridine orange-stained cell culture 120 hours post inoculation with wart specimen from DI. (x800)

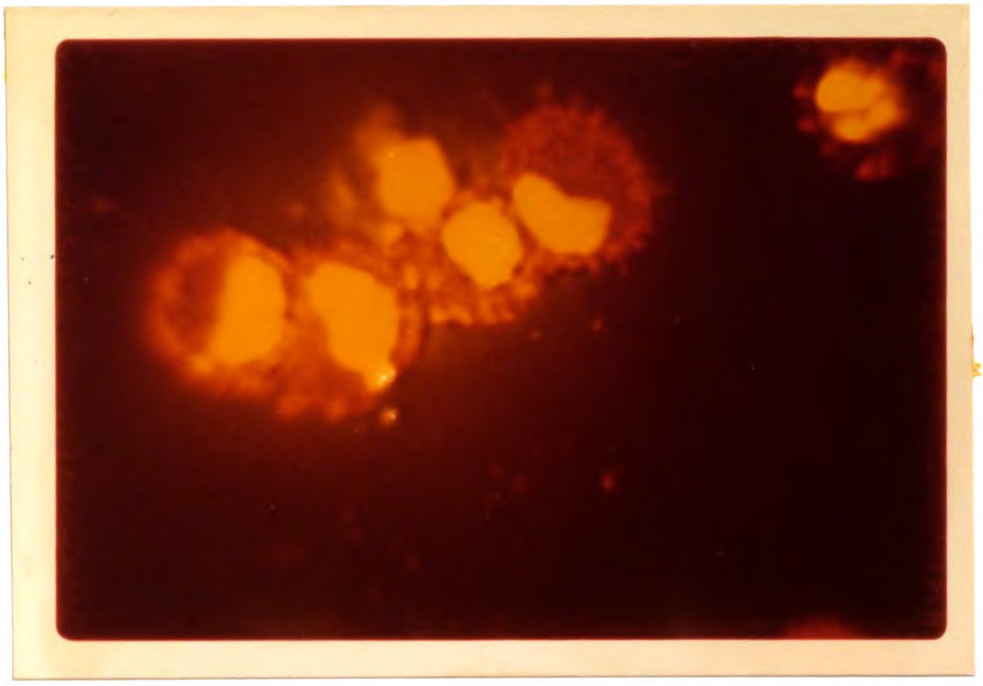

Figure 14. Acridine orange-stained cell culture from second serial passage of wart virus DI. (48 hours post inoculation) (x800)

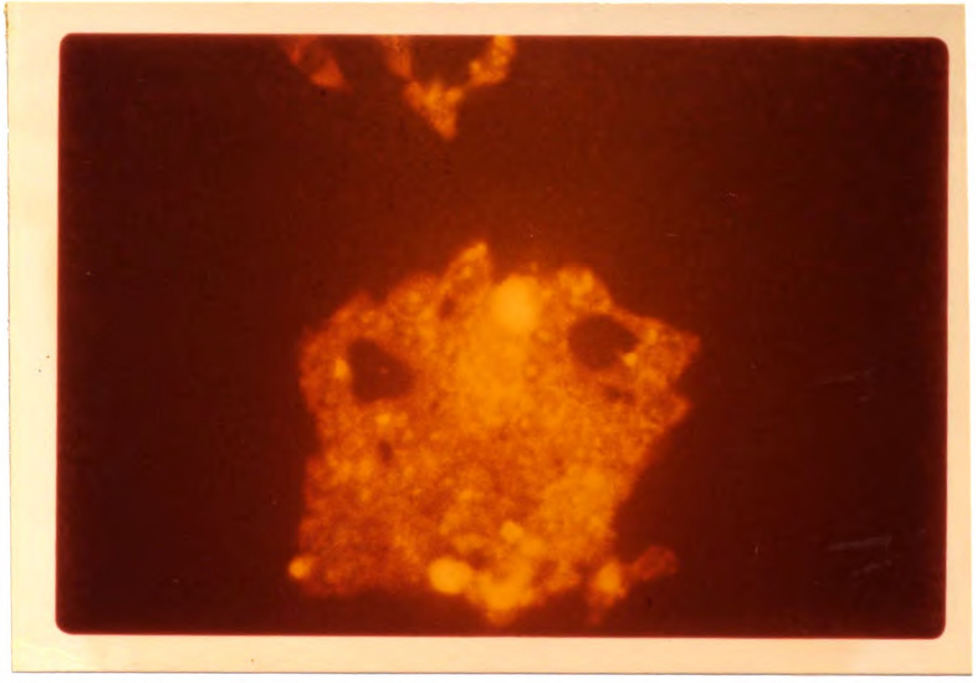

Figure 15. DNase digested and acridine orange-stained cell culture from second passage of wart virus DI. (48 hours post inoculation) (x200)

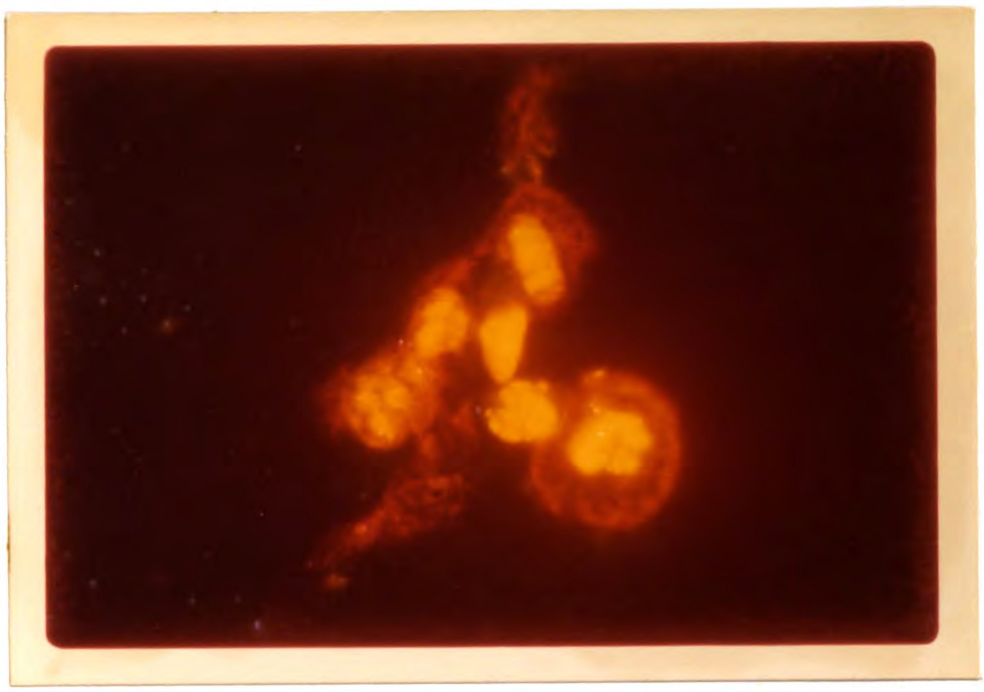

Figure 16. Acridine orange-stained infected cell culture from third serial passage of wart virus DI. (24 hours post inoculation) (x800)

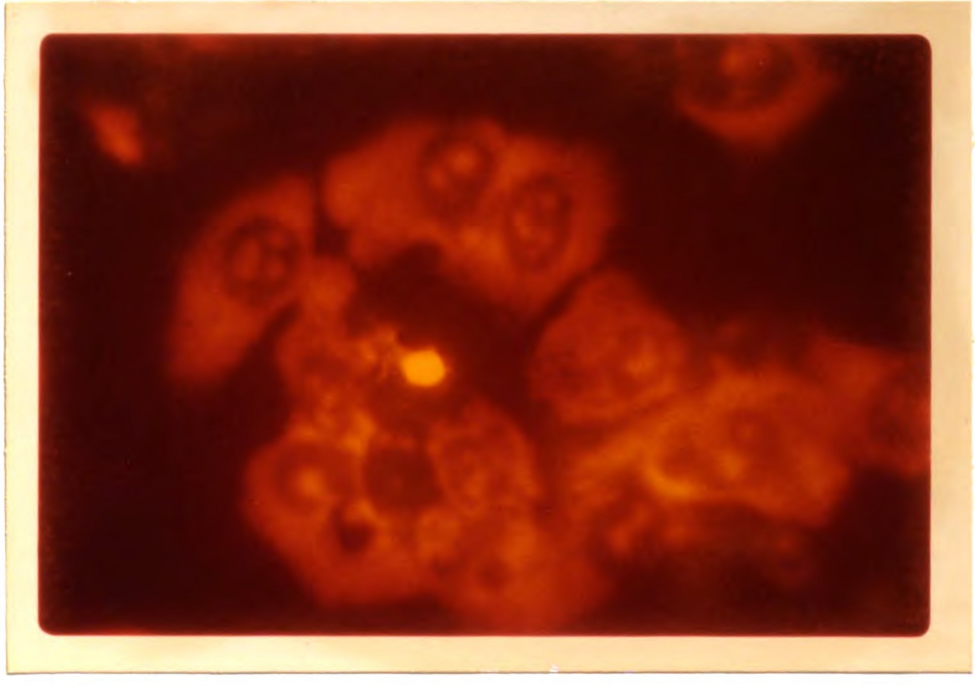

Figure 17. DNase digested and acridine orange-stained cell culture from third serial passage of wart virus DI. (24 hours post inoculation) (x800)

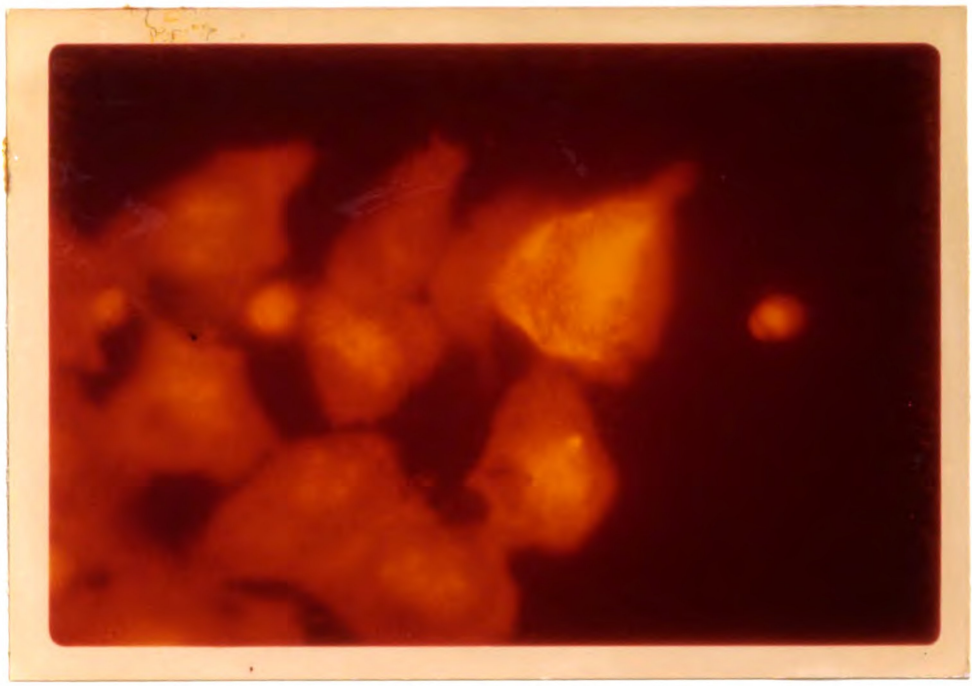

Figure 18. DNase digested and acridine orange-stained cell culture from third serial passage of wart virus DI. (24 hours post inoculation) (x800)

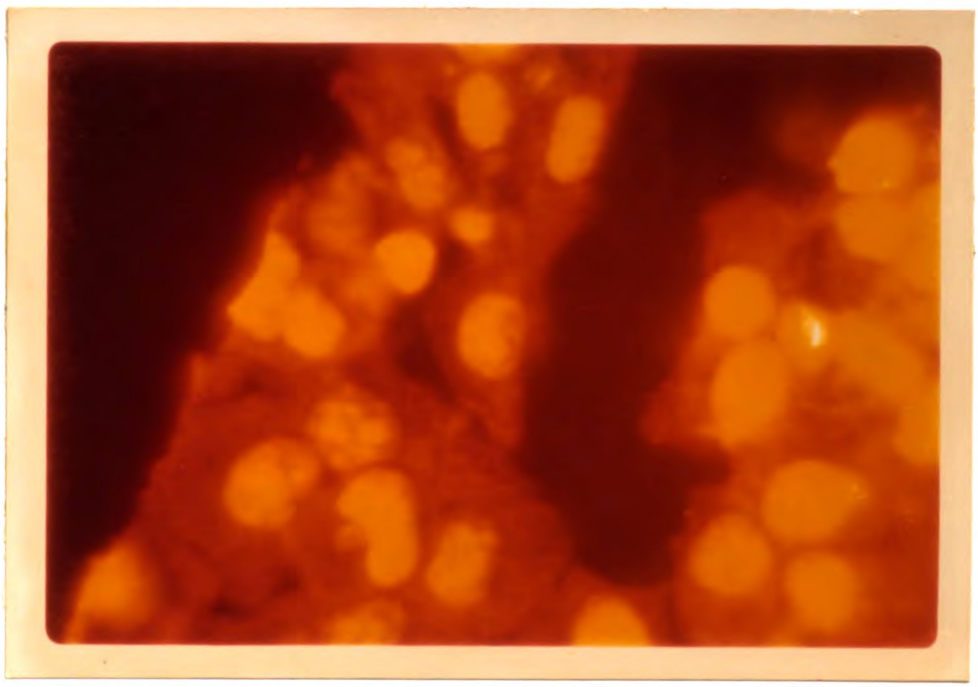

Figure 19. Acridine orange-stained cell culture from fourth passage of wart virus DI. (24 hours post inoculation) (x800)

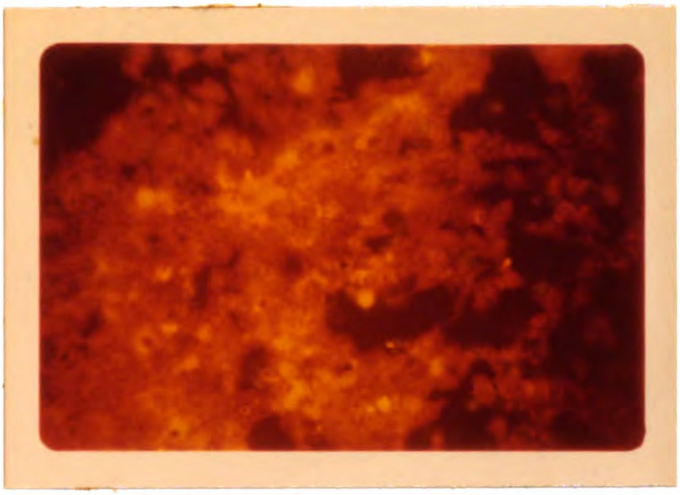

Figure 20. DNase digested and acridine orange-stained cell culture from fifth passage of wart virus DI. (72 hours post inoculation) (x100)

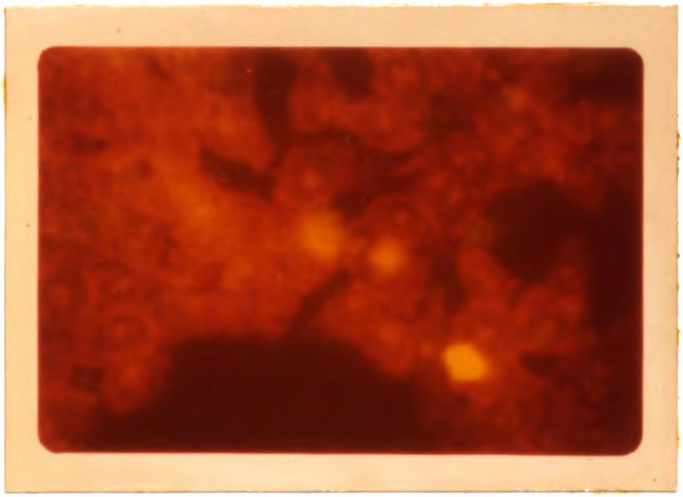

Figure 21. Same as Figure 20 at higher magnification. (x400)

Table 1. Presence in infected cultures of intranuclear DNA staining inclusions resistant to DNase digestion.

Inoculum	Time Post Inoculation of Fixation				
	24 hours	48 hours	72 hours	96 hours	120 hours
Uninfected control	-	-	-	-	-
GB wart tissue	-	+	+/-	++	++
SD wart tissue	-	+	+	+/-	++
DI wart tissue	+	+	+/-	++	+++
MEF wart tissue	-	+/-	n.d.	++	++/-
1st passage cells	-	n.d.	+	n.d.	n.d.
2nd passage cells	+/-	++	n.d.	n.d.	+++
3rd passage cells	+	n.d.	+/-	+++	n.d.
4th passage cells	+	n.d.	++	n.d.	+++
5th passage cells	+	n.d.	++/-	n.d.	++++

Key: n.d. - study not done
+ Presence of intranuclear inclusion in 1-2% of monolayer
++ Presence of intranuclear inclusion in 5-10% of monolayer
+++ Presence of intranuclear inclusion in 10-15% of monolayer
++++ Presence of intranuclear inclusion in 15-20% of monolayer
- Absence of inclusions in monolayer

Figures 15, 17, 20, and 21, the nuclei appear to be enlarged and rounded. Of particular interest is Figure 18. The nucleus of one of the cells in the field has become greatly distorted and contains a large amount of particulate DNA fluorescing material, much like that in the cells previously seen in Figure 12. Here again the results do not present evidence of the replication cycle of the virus, as the amount of virus being transferred into the cultures has not been standardized in any way. Consideration must also be made of the variance in the individual cell susceptibility. The findings merely support the fact that an agent is being transferred in serial cultures which is capable of producing effects similar to those seen upon primary isolation.

III. Results of Fluorescent Antibody Studies

AU tissue cell cultures had previously been grown in growth media supplemented with twenty per cent human serum. Prior to use they were adapted to growth in twenty per cent fetal calf serum since, as pointed out by Murphy and Furtado (1963), the cells tend to adsorb substances from the added human serum which cannot be removed even by careful washing. Further evidence of this was revealed by an experiment in which uninfected cultures grown in media supplemented with human serum were overlayed with the fluorescein conjugated rabbit antihuman globulin, but not subjected to prior treatment with specific antisera. When these cells were observed in the fluorescence microscope with ultraviolet light, they exhibited a large amount of fluorescence in some cases almost of the intensity emitted by infected cell cultures grown in the media supplemented with calf serum. This fluorescence was not visible in cultures prior to treatment with the conjugated antiglobulin. Thus the cells in the former cultures must

have adsorbed materials from the human serum which were now reacting with the labeled antiserum, thereby causing the cells to fluoresce when observed.

The results of fluorescent antibody tests of cultures inoculated with freshly removed wart tissues are shown in Figures 22 through 28. Viral antigen was detectable in the nuclear area of the cells within 48 hours after inoculation as evidenced by large bright fluorescent clumps in Figures 22 and 24. When cultures were observed at 72 hours post inoculation, in addition to the bright fluorescent clumps, a more diffuse and particulate (small fluorescent particles) fluorescence was visible in the cytoplasm of some of the cells as seen in Figures 23 and 25. However, this particulate fluorescence could be detected in only 1 or 2 per cent of the cells in the monolayer, while large bright fluorescent clumps were visible in about 5 per cent of the cells. Both types of specific fluorescence was also found to be present in 10 to 20 per cent of the cells at 96 and 120 hours after inoculation (Figures 26 and 27). The observation of controls with the fluorescent microscope showed that either very little or no fluorescence was present. In Figure 28 is shown a culture five days after inoculation which was treated with normal serum instead of the specific antiserum. It is readily seen that though some fluorescence is present, it is of very low intensity. Other controls demonstrated either this type of fluorescence or one of much lower intensity.

Cultures inoculated with subcultured material also demonstrated specific fluorescence. An example is presented in Figure 29, in which the cells display the large intranuclear clumps of fluorescent protein as well as a small number of fluorescent particles throughout the cytoplasm.

The results of the fluorescent antibody studies conducted with cultures inoculated with fragments of wart materials frozen from the time of their removal in 1957 are shown in Figures 30 and 31.

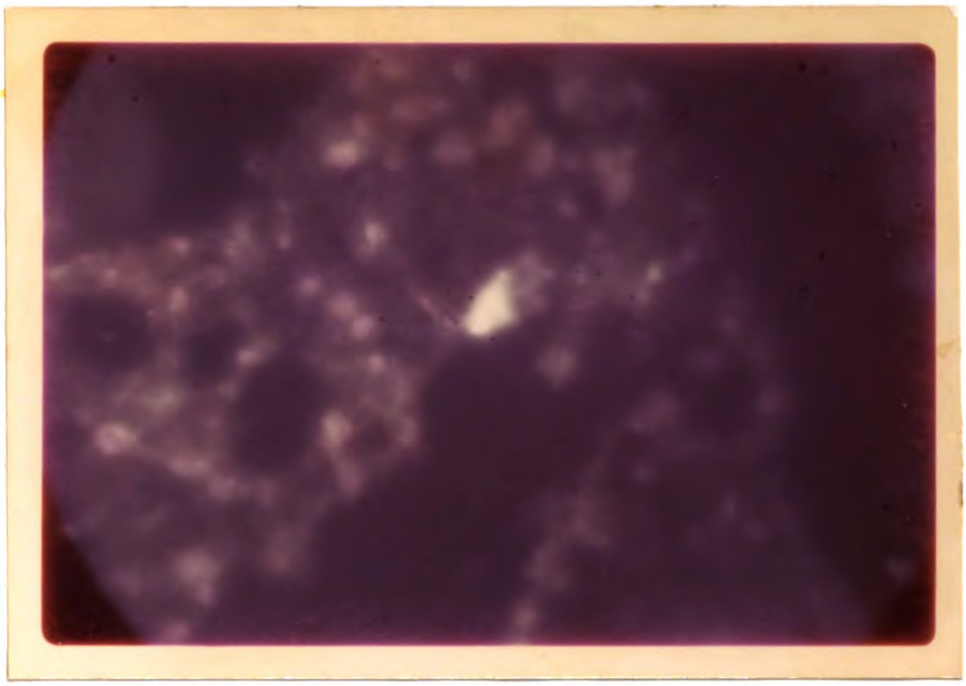

Figure 22. Fluorescent antibody study of cell culture 48 hours post inoculation with wart tissue from DI. (x400)

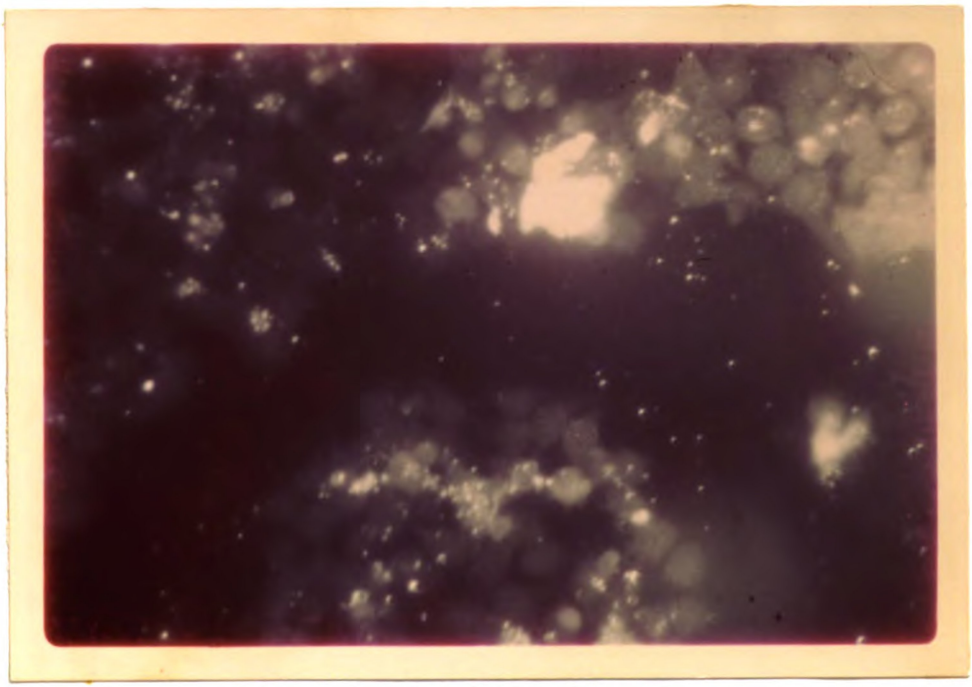

Figure 23. Fluorescent antibody study of cell culture 72 hours post inoculation with wart tissue from DI. (x400)

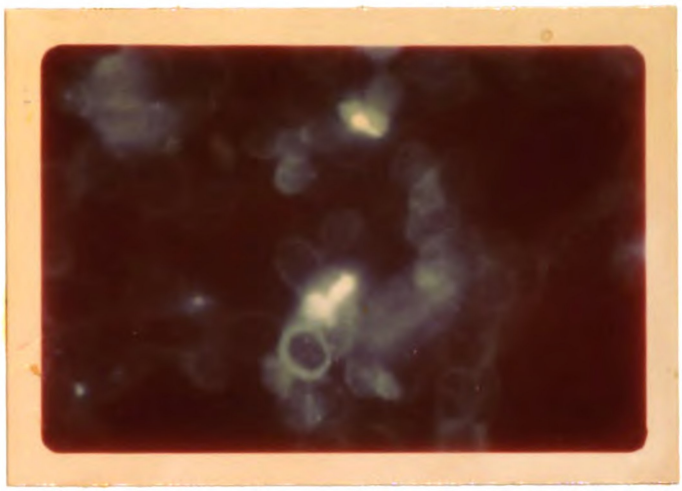

Figure 24. Fluorescent antibody study of cell culture 48 hours post inoculation with wart tissue from MEF. (x400)

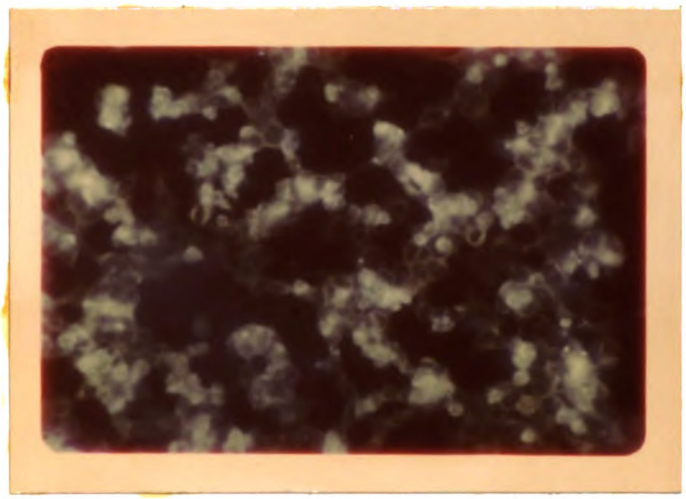

Figure 25. Fluorescent antibody study of cell culture 72 hours post inoculation with wart tissue from MEF. (x200)

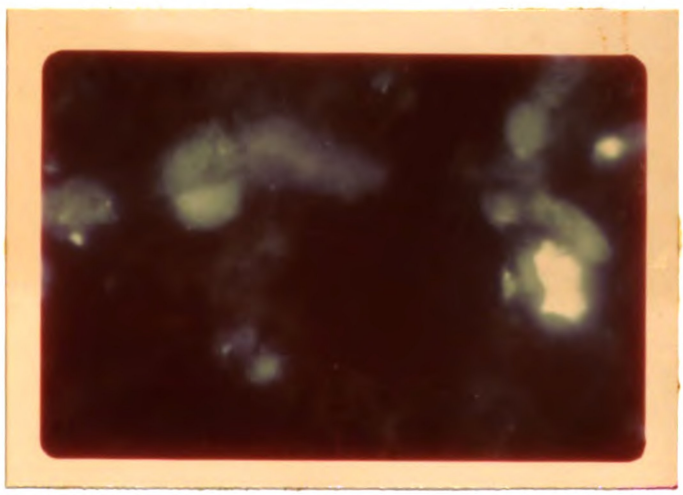

Figure 26. Fluorescent antibody study of cell culture 96 hours post inoculation with wart tissue from MEF. (x400)

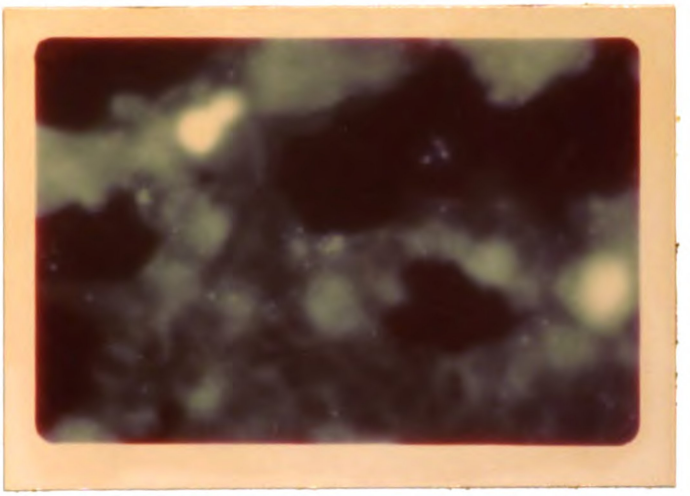

Figure 27. Fluorescent antibody study of cell culture 120 hours post inoculation with wart tissue from MEF. (x400)

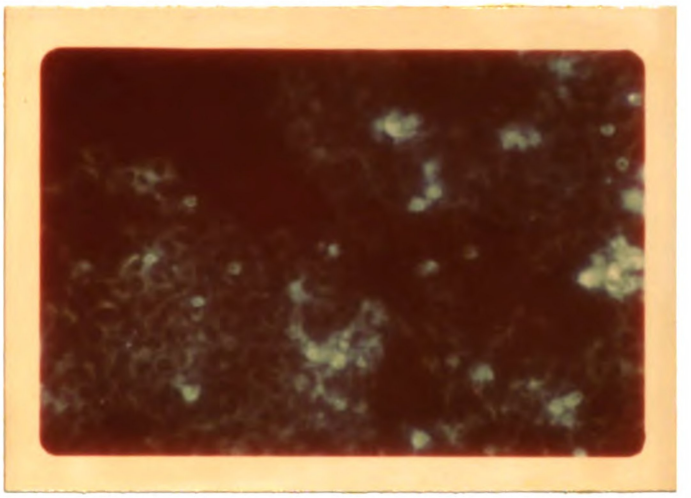

Figure 28. Fluorescent antibody study with normal serum control of cell culture 120 hours post inoculation with wart tissue from MEF. (x200)

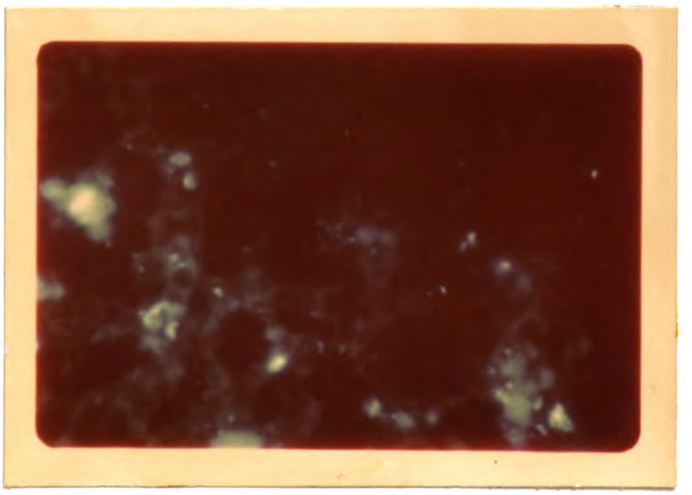

Figure 29. Fluorescent antibody studies of cell culture inoculated with third passage of wart virus FR. (48 hours post inoculation) (x200)

Figure 30. Fluorescent antibody studies of cell culture inoculated with frozen wart material from RD. (x400)

Figure 31. Fluorescent antibody studies of cell culture inoculated with frozen wart material from HL. (x400)

These are similar to the cell cultures observed after inoculation with warts which had been stored in glycerol for 2 or 3 years at 4° C. Cell cultures inoculated with these tissues did not exhibit a cytopathic effect even after periods of two or three weeks of observation. Although specific fluorescence of the cells was evident, it was not of the intensity found in cells inoculated with fresh wart tissue or cells from subcultures. Perhaps the virus in the tissues which had been stored was still infective but unable to undergo replication.

Of the specific antisera utilized in these experiments two, JP and DA, had been found to have detectable complement fixing antibodies (Russell, 1961). Others, JH, CD, and GH, had no such antibodies. By the indirect technique both types of antisera gave positive fluorescent antibody reactions of comparable intensity, while normal sera from individuals with no previous history of warts gave either a reaction of very low intensity or none at all.

The results of the fluorescent antibody studies on the various cultures have been summarized in Table 2.

Table 2. Intensity of specific fluorescence in cells infected with the wart virus.

Inoculum	Time Post Inoculation of Fixation				
	24 hours	48 hours	72 hours	96 hours	120 hours
Controls	-	-	-	-	-
DI wart tissue	-	+1	+4 +2	+4 +3	n.d.
MEF wart tissue	-	+1	+4 +2	+4 +3	+4 +3
3rd passage of FR	-	+2 +4	+4 +2	n.d.	n.d.
Glycerinated warts	-	+1	+4	n.d.	n.d.
Frozen warts	-	+1	+4	n.d.	n.d.

Key: n.d. study not done
+1 Presence of intranuclear fluorescence in 1-2% of the monolayer
+2 Presence of intranuclear fluorescence in 5-10% of the monolayer
+3 Presence of intranuclear fluorescence in 10-20% of the monolayer
+4 Presence of large amounts of intranuclear fluorescence and also diffuse particulate fluorescence
- Absence of any fluorescence

DISCUSSION

It is accepted today that the etiological agent of the common human wart is a virus. Numerous electron micrographs have shown the virus in tissue and in extracts. However, few workers have been successful in the isolation of the virus in vitro. Mendelson and Kligman (1961) established that the agent which they isolated was the wart virus with the production of wart growths in volunteers injected with cell-free nutrient fluids. This has not been possible with the agent isolated in this laboratory by Hayashi (1961), since the virus can be transmitted in serial passage only by the use of cells from infected cultures as an inoculum. It would be very dangerous indeed to attempt the inoculation of humans with material from the infected cell cultures of human skin. However, the results of fluorescent antibody and acridine orange studies with infected cultures presented here provide additional evidence that a viral agent has been isolated from wart tissues on four consecutive occasions.

Similar difficulties have been encountered in isolating the agents from other diseases of viral etiology. Upon initial isolation the virus of cytomegalic inclusions also required the transfer of cells for passage. Experiments conducted by McAllister et al. (1963) utilizing acridine orange staining and the fluorescent antibody technique showed intra-nuclear and intracytoplasmic lesions in the cells. Even with subsequent cell degeneration apparently releasing the virus, the supernatant fluids showed no virus. After subsequent passage, it was found that the virus was released into the nutrient fluids, but it proved to have a half life of less than one hour at 37^{o}C. Perhaps this may also be true of the wart

virus. Degenerating cell nuclei have been seen which appeared to be releasing virus which seemed to be in a mature state covered with its protein coat as it resisted DNase digestion and the viral protein could be detected with the fluorescent antibody technique. Perhaps the wart virus is also extremely thermolabile, and unless it is immediately adsorbed by neighboring uninfected cells, it quickly degenerates. Perhaps the desmosomes seen by Chapman et al. (1963) play a role in the intercellular transfer of the virus.

As the ultraviolet microscope allows resolution of particles with diameters of 0.1 micron, by utilizing the sensitive acridine orange technique (estimated by Mayor and Diwan (1961) to resolve 50 closely packed small RNA virus particles compared to the fluorescent antibody technique which has a limit of 5×10^{-15} grams of antigen (Coon, 1958) which corresponds to some hundreds of similar virus particles) Williams (1961) was able to detect the wart virus in cutaneous lesions. The intranuclear inclusion bodies of the upper rete layer and the nuclei of the horny layer fluoresced yellow-green and this fluorescence was resistant to DNase digestion. The DNA nature of the wart virus was confirmed by Noyes (1964) with the acridine orange staining of purified preparations. The agent isolated in these studies met these requirements, since it contained the nucleic acid DNA and appeared to undergo replication only in the nuclei of the infected cells. The fact that not all the cells elicited the same type of response, some demonstrating enlarged rounded nuclei while others demonstrated shrunken distorted nuclei, might be due to the heterogeneous cell population of the cell line. Since the cultures were not derived from a single clone, they may respond differently upon infection. Still, the agents from the tissues of the four different patients produced similar cytopathic effects upon initial isolation and after subculturing.

Two other tumor producing agents, SV-40 (Mayor, 1962) and polyoma (Williams and Sheinin, 1961), have also been shown to be DNA viruses which undergo replication in the nucleus of the infected cell. Virus isolated from molluscum contagiosum, one of the epidermal tumors of man, has also proved to be of DNA composition (Raskin, 1962); however, it appeared to undergo maturation only in the cytoplasm. Although it is impossible to really make a comparison of these viruses with the viruses isolated in the experiments presented here, there are similarities which cannot be overlooked.

In fluorescent antibody studies of two of the epidermal tumors of man, warts and molluscum contagiosum, Epstein et al. (1963) detected the antigens of molluscum contagiosum but not those of warts. Perhaps the wart virus exists in the tissues in early stages of development and is not detectable, since the nucleic acid is the infectious material and the protein coat merely serves to maintain transmissibility. This was the explanation offered by Noyes and Mellors (1957) when they found that the papillomas of demesticated rabbits exhibited less fluorescence than wild rabbits. When concentrating extracts of over 100 warts by sucrose gradient centrifugation, Noyes (1964) indeed found only a thin band of intact particles with well preserved structures. Other bands consisted either of particles devoid of internal material or elongated, enlarged and dumbell forms of protein coats. Thus it seems that warts contain only small numbers of complete infectious and antigenic virus. However, once the virus is exposed to uninfected cell cultures these few infective virus particles may be transferred as the tissue degenerates. Once they have infected the cells synthesis of the viral protein coat ensues.

In the experiments reported here, this was found to be true. At 48 hours post inoculation at least one or two per cent of the monolayer exhibited specific intranuclear fluorescence when reacted with

specific antiserum and the conjugated antihuman globulin. As time progressed the fluorescent antibody technique also detected diffuse fluorescent particles in the cytoplasm; however, acridine orange studies did not show the presence of such cytoplasmic distributions of viral DNA. Perhaps these may be noninfectious protein coats formed in excess by the cell. This theory is supported by the observations of Noyes (1964) of single and aggregated particles devoid of internal material in purified virus preparations. Russell (1961) found that the cell-free fluids from infected AU cell cultures which did not infect other cultures, were antigenic in rabbits. The rabbit antisera reacted both with cells and concentrated antigen from wart tissues.

The exact mechanism of the immune response to the wart virus is unknown. The antisera from some patients with warts do contain antibodies which can be detected by neutralization (Hayashi, 1961) and complement fixation (Russell, 1961) tests. It has been shown that the fluorescent antibody technique is a much more sensitive method of antibody detection. Raskin (1963) found that fluorescent antibodies could be detected in molloscum contagiosum patients, while the presence of neutralizing antibodies could not be demonstrated. As pointed out by Riggs and Brown (1962) specific antisera may be diluted to eliminate non-specific reactions and they will still give strong positive fluorescent antibody reactions while other antigen-antibody reactions, i.e., neutralization and complement fixation, were no longer present.

Therefore, the results with antisera utilized in these experiments which contained no detectable complement fixing antibodies seem quite reasonable. The level of antibodies may be low due to the small amount of antigen available for the stimulation of antibody response in the infected individual. Since the virus has been found only intracellularly in electron micrographs of the wart tissues, it is not readily exposed to the antibody-forming systems of the host. Thus it would appear likely

that only small amounts of antibody would be formed to the viral antigen. These antibodies could only be detected by extremely sensitive techniques, as previously stated the fluorescent antibody technique can detect 5×10^{-15} grams of antigen. As shown by Almeida et al. (1963) one antibody molecule links two univalent wart virus antigens. This antigen-antibody reaction thus requires twice as much antigen as antibody. Thus it is possible to detect small amounts of antibody as well as antigen.

Perhaps with the development of even more refined techniques, it may be possible not only to detect the presence of the specific viral antibodies but also gain information concerning their role in the formation and regression of warts.

SUMMARY

1. Acridine orange staining of monolayer cultures infected with freshly removed wart tissues from 4 different patients or with cells from infected subcultures showed the presence of intranuclear inclusions of DNA within 24 to 48 hours post inoculation.

2. The number of cells containing these inclusions increased as time progressed until about 10 to 20 per cent of the cells were infected by 5 days at which time the cell monolayer was degenerating.

3. The inclusions were not susceptible to RNase digestion and were removed by DNase digestion only when the cells were first treated with pepsin.

4. Clusters of viral protein were detected by the fluorescent antibody reaction in the nuclei of infected cells (inoculum was freshly removed wart fragments) 48 hours post inoculation.

5. The number of cells exhibiting fluorescence increased to 10 or 20 per cent as time elapsed. In some cases diffuse fluorescent viral protein particles were detectable in the cytoplasm of infected cells.

6. Specific fluorescence was also exhibited in the nuclei of cells infected by inoculation with cells from infected subcultures, frozen wart tissue or warts stored in glycerol at $4^{\circ}C$.

7. Sera previously found to have detectable complement fixing antibodies or which had no such demonstratable antibodies were found to give a positive fluorescent antibody reaction. Sera from patients with no known history of warts gave a negative reaction or one of much lower intensity.

BIBLIOGRAPHY

ALMEIDA, J. D., A. F. HOWATSON, AND M. G. WILLIAMS 1962 Electron microscope studies of human warts, sites of virus production and nature of the inclusion bodies. J. Invest. Dermat., 38, 337-345.

ALMEIDA, J. D., B. CINADER, AND A. HOWATSON 1963 The structure of antigen-antibody complexes. J. Exptl. Med., 118, 327-340.

ANDERSON, E. S., J. A. ARMSTRONG, AND J. S. NIVEN 1959 Fluorescence microscopy: observations of virus growth with aminoacridines. Symp. Soc. Gen. Microbiol., 9, 224-254.

ARMSTRONG, J. A. 1956 Histochemical differentiation of nucleic acids by means of induced fluorescence. Exptl. Cell Research, 11, 640-642.

ARMSTRONG, J. A. AND J. S. NIVEN 1957 Histochemical observations on cellular and viral nucleic acids. Nature, 180, 1335-1336.

ARMSTRONG, J. A. AND P. K. HOPPER 1959 Fluorescence and phase-contrast microscopy of human cell cultures infected with adenoviruses. Exptl. Cell Research, 16, 584-594.

ARWYN, C. 1960 Electron microscope observations on human wart. Dermatologica, 121, 193-203.

BEARD, J. W. AND J. G. KIDD 1936 Antigenic individuality of certain papilloma viruses. Proc. Soc. Exptl. Biol. Med., 34, 451-453.

BIVINS, J. A. 1953 The growth in the developing chicken embryo of a filterable agent from Verruca Vulgaris. J. Invest. Dermat., 20, 471-481.

BLANK, H., M. BUERK, AND F. WEIDMAN 1951 The nature of the inclusion body of Verruca Vulgaris: A histochemical study of nucleotides. J. Invest. Dermat., 16, 19-31.

BLANK, H. AND G. RAKE 1955 Viral and rickettsial diseases of the skin, eye, and mucous membranes of man, pp. 156-181 Little, Brown, and Company, Boston.

BRADLEY, D. F. AND G. FELSENFELD 1959 Aggregation of acridine dye on native denatured deoxyribonucleotides. Nature, 184, 1920-1922.

BROWN, E. R. AND J. J. BITTNER 1961 Fluorescent antibody reactions against the mouse mammary tumor agent. Proc. Soc. Exptl. Biol. Med., 106, 303-306.

BUNTING, H. 1953 Close-packed array of virus-like particles within cells of human skin papillomas. Proc. Soc. Exptl. Biol. Med., 84, 327-332.

BUNTING, H., M. J. STRAUSS, AND W. G. BANFIELD 1952 The cytology of skin papillomas that yield virus-like particles. Am. J. Path., 38, 985-1002.

CHADWICK, C. S., M. G. McENTEGART, AND R. C. NAIRN 1958 Fluorescein protein tracers, a simple alternative to fluorescein. Lancet, 412-414.

CHAPMAN, G. B., L. M. DRUSIN, and J. E. TODD 1963 Fine structure of the human wart. Am. J. Path., 42, 614-642.

CIUFFO, G. 1907 Innesto positivo con filtrato di Verruca Volgare. Gior. Ital. d. mal. vener., 48, 12-17.

COHEN, S. M., I. GORDON, F. RAPP, J. C. MACAULAY, AND S. M. BUCKLEY 1955 Fluorescent antibody and complement fixation tests of agents isolated in tissue culture from measles patients. Proc. Soc. Exptl. Biol. Med., 90, 118-122.

COONS, A. H., H. J. CREECH, AND R. N. JONES 1941 Immunological properties of antibodies containing a fluorescent group. Proc. Soc. Exptl. Biol. Med., 47, 200-202.

COONS, A. H., H. J. CREECH, R. N. JONES, AND E. BERLINER 1942 The demonstration of pneumococcal antigen in tissues by use of fluorescent antibody. J. Immunol., 45, 159-170.

COONS, A. H. AND M. H. KAPLAN 1950 Localization of antigen in tissue cells. II. Improvement in a method for the detection of antigen by means of fluorescent antibody. J. Exptl. Med., 91, 1-13.

COONS, A. H., J. C. SNYDER, F. S. CHEEVER, AND E. S. MURRAY 1950 Localization of antigens in tissue cells. IV. Antigens of rickettsiae and mumps virus. J. Exptl. Med., 91, 31-38.

COONS, A. H. 1958 Fluorescent antibody methods. In: General Cytochemical Methods, J. F. Daneilli, ed. pp. 399-422. Academic Press Inc., New York.

DART, L. H. AND T. R. TURNER 1959 Fluorescence Microscopy in exfoliative cytology. Lab. Invest., 8, 1513-1522.

EPSTEIN, W. L., I. SENECAL, H. KRASNOBROD, AND A. M. MASSING 1963 Viral antigens in human epidermal tumors: Localization of an antigen to molluscum contagiosum. J. Invest. Dermat. 40, 51-59.

FELSHER, S. B. 1947 Virus studies on cutaneous material. Inoculation of the chick chorioallantoic membrane with material from cutaneous lesions. J. Invest. Dermat., 8, 123-124.

FRANKLIN, R. M. 1962 A cytochemical description of the multiplication of mengovirus in L-929 cells. J. Cell Biol., 12, 1-15.

FRASER, K. B. AND M. GHARPURE 1962 Immunofluorescent tracing of polyoma virus in transformation experiments with BHK 21 cells. Virology, 18, 505-507.

FURUSAWA, E. AND W. CUTTING 1960 Detection of Viral DNA in Inclusion Bodies of Ectromelia infected tumor cells in fluorescent microscopy. Virology, 11, 632-634.

GOLDSCHMIDT, H. AND A. M. KLIGMAN 1958 Inoculation of humans with ectodermatrophic viruses. J. Invest. Dermat. 31, 175-179.

GOLDSTEIN, M. N., R. HIRAMOTO, AND D. PRESSMAN 1959 Comparative fluorescein labeling and cytotoxicity studies with human cell strains HeLa, Raos and 407-Liver and with fresh surgical specimens of cervical carcinoma, osteogenic sarcoma, and normal adult liver. J. Nat. Cancer Inst., 22, 697-706.

HANKS, J. H. AND R. E. WALLACE 1949 Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exptl. Biol. Med., 71, 196-200.

HAYASHI, HAJIME 1961 Studies on the viral etiology of Verruca Vulgaris in tissue cell culture. Ph. D. Thesis Michigan State University. East Lansing, Mich.

HORNE, R. W. AND P. WILDY 1961 Symmetry in virus architecture. Virology, 15, 348-373.

JADASSOHN, J. 1896 Sind die Verruca Vulgaris übertrag bar? Verhandl. dermat. gesellsch., 5, 497.

KINGERY, L. B. 1921 The etiology of common warts. Their production in the second generation. J. Am. Med. Assoc., 76, 440-442.

LIPSCHÜLZ, B. 1924 Zur kenntnis der atiolgie und der strukturellen architecture die warze. Arch. Derm. u. Syph. Wien., 148, 201.

LIU, C. 1956 Rapid diagnosis of human influenza from nasal smears by means of fluorescein-labeled antibody. Proc. Soc. Exptl. Biol. Med., 92, 883-887.

LOH, P. H. AND J. L. RIGGS 1961 Demonstration of the sequential development of vaccinial antigens and virus in infected cells. Observations with cytochemical and differential fluorescent procedures. J. Exptl. Med., 114, 149-160.

LOUIS, C. J. 1958 The significance of cell type in fluorescein globulin staining tissues. Brit. J. Cancer, 12, 537-546.

MALMGREM, R. A., M. A. FINK, AND W. MILLS 1960 Demonstration of the intracellular location of Rous Sarcoma virus antigen fluorescein-labeled antisera. J. Natl. Cancer Inst., 24, 995-999.

MALMGREM, R. A., G. RABOTTI, AND A. S. RABSON 1960a Intracellular localization of polyoma virus antigen demonstrated with fluorescein-labeled antibody. Virology, 12, 361-363.

MARRACK, J. 1934 The nature of antibodies. Nature, 292-293.

MATTERN, C. F., A. C. ALLISON, AND W. P. ROWE 1963 Structure and comparison of K virus and its relation to the "papova-virus" group. Virology, 20, 413-419.

MAYOR, H. D. 1961 Cytochemical and fluorescent antibody studies on the growth of poliovirus in tissue cultures. Texas Repts. Biol. Med., 19, 106-122.

MAYOR, H. D. 1961a Acridine Orange staining of purified polyoma virus. Proc. Soc. Exptl. Biol. Med., 108, 103-105.

MAYOR, H. D. AND A. R. DIWAN 1961 Studies on the acridine orange staining of two purified RNA viruses: poliovirus and TMV. Virology, 14, 74-82.

MAYOR, H. D. AND N. O. HILL 1961 Acridine orange staining of a single stranded DNA bacteriophage. Virology, 14, 264-266.

MAYOR, H. D., S. E. STINEBAUGH, R. M. JAMISON, L. E. JORDAN, AND J. L. MELNICK 1962 Immunofluorescent, cytochemical, and microcytological studies on the growth of simian vacuolating virus in tissue culture. Exptl. and Molecular Path., 1, 397-416.

MAYOR, H. D. 1963 The nucleic acids of viruses as revealed by their reactions with fluorochrome acridine orange. Internatl. Rev. Exptl. Path., 2, 1-45.

MAYOR, H. D., R. M. JAMISON, AND L. E. JORDAN 1963 Biophysical studies on the nature of the simian popova virus particle (Vacuolating SV-40 virus). Virology, 19, 359-366.

McALLISTER, R. M., R. S. STRAW, J. E. FILBERT, AND G. R. GOODHART 1963 Human cytomegalovirus. Cytochemical observations of intracellular lesion development correlated with viral synthesis and release. Virology, 19, 521-531.

MENDELSON, C. G. AND A. M. KLIGMAN 1961 Isolation of wart virus in tissue culture. Arch. Dermatol., 83, 559-562.

MURPHY, W. H. AND D. FURTADO 1963 Isolation of viruses from children with acute leukemia. University of Mich. Medical Bulletin, Ann Arbor, Mich.

NOYES, W. F. 1955 Visualization of Egypt 101 virus in the mouse brain and in cultured human carcinoma cells by means of fluorescent antibody. J. Exptl. Med., 102, 243-248.

NOYES, W. F. AND R. C. MELLORS 1957 Fluorescent antibody detection of the antigens of the Shope papilloma virus in papillomas of the wild and domestic rabbits. J. Exptl. Med., 106, 555-562.

NOYES, W. F. 1959 Studies of the Shope rabbit papilloma virus. II. The localization of infective virus in papillomas of the cottontail rabbit. J. Exptl. Med., 109, 423-428.

NOYES, W. F. 1964 Structure of human wart virus. Virology, 23 65-72.

OGILVIE, R. F. 1951 Pathological Histology, 4th ed. pp. 103-104. Williams and Wilkins Co., Baltimore.

PITAL, A. AND S. L. JANOWITZ 1963 Enhancement of staining intensity in the fluorescent antibody reaction. J. Bact., 86, 888-889.

RAPP, R. AND M. B. MELNICK 1963 Plaque assay for measurement of cells infected with zoster virus. Science, 141, 433-434.

RASKIN, J. 1963 Molluscum contagiosum. Tissue culture and serological study. Archiv. Dermat., 87, 552-559.

RHIM, J. S., L. E. JORDAN, AND H. D. MAYOR 1962 Cytochemical, fluorescent-antibody and electron microscope studies on the growth of reovirus (Echo 10) in tissue culture. Virology, 17, 342-355.

RIGGS, J. L., R. J. SEIWALD, J. J. BURCHHALTER, C. M. DOWNS, AND T. G. METCALF 1958 Isothiocyanate compounds as fluorescent labeling agents for immune serum. Am. J. Path., 34, 1081-1097.

RIGGS, J. L., AND G. C. BROWN 1962 Application of direct and indirect immunofluorescence for identification of enteroviruses. Proc. Soc. Exptl. Biol. Med., 110, 833-837.

ROBERTSON, H. E., K. T. BRUNNER, AND J. T. SYVERTON 1955 Propagation in vitro of poliomyetlitis viruses. III. pH change of HeLa cell culture for assay. Proc. Soc. Exptl. Biol. Med., 88, 119-122.

RUSSELL, J. W. 1961 Complement Fixation Studies with Verruca Vulgaris. M. S. Thesis Michigan State University, East Lansing, Mich.

SCHÜMMELFEDER, N. 1958 Histochemical significance of the polychromatic fluorescence induced in tissues stained with acridine orange. J. Histochem. Cytochem., 6, 392-393.

SERRA, A. 1908 Richerche istologiche experimentali sul. condiloma acumenato. I. papillomi del capo e la verruca volgare. Gior. Ital. d. mal. vener. 49, 11.

SIEGEL, B. V. AND F. G. NOVY 1955 Cultivation studies on wart suspension of Verruca Vulgaris and Condyloma Acuminatum. J. Invest. Dermat., 25, 265-268.

SIEGEL, B. V. 1956 Contaminant filterable agent derived from a human wart. J. Invest. Dermat., 27, 379-381.

SIEGEL, B. V. 1960 Electron microscope studies of the virus of human warts. J. Invest. Dermat., 35, 91-93.

SILVERSTEIN, A. M. 1957 Contrasting fluorescent labels for two antibodies. J. Histochem., 5, 94-95.

SMITH, C. W., J. D. MARSHALL, AND W. C. EVELAND 1959 Use of contrasting fluorescent dye as a counterstain in fixed tissue preparations. Proc. Soc. Exptl. Biol. Med., 102, 179-181.

SOUTHAM, C. M., W. F. NOYES, AND R. MELLORS 1958 Virus in human cancer cells in vivo. Virology, 5, 395-400.

STEINER, R. F. AND R. F. BEERS 1958 Spectral changes accompanying binding of acridine orange by polyadenylic acid. Science, 127, 335.

STONE, R. S., R. E. SHOPE, AND D. H. MOORE 1959 Electron microscope study of development of the papilloma virus in the skin of the rabbit. J. Exptl. Med. 110, 543-546.

STRAUSS, M. J., E. W. SHAW, H. BUNTING, AND J. L. MELNICK 1949 Crystalline virus-like particles from skin papillomas characterized by intranuclear inclusion bodies. Proc. Soc. Exptl. Biol. Med., 72, 46-50.

STRAUSS, M. J., H. BUNTING, AND J. L. MELNICK 1950 Virus-like particles and inclusion bodies in skin papillomas. J. Invest. Dermat., 15, 434-443.

STRAUSS, M. J., H. BUNTING, AND J. L. MELNICK 1951 Eosinophilic inclusion bodies and cytoplasmic masses in verrucae. J. Invest. Dermat., 17, 209-211.

STRUGGER, S. 1940 Fluorezenzmikroskopische untersuchungen über die aufnahme und spercherung des acridinorange durch lebende und tote pflanzennellen. Jena Z. Naturw., 73, 97.

ULBRICH, A. P., N. W. ARENDS, AND D. KOPRINCE 1957 Warts: Discussion with special reference to therapy by total enucleation. J. Am. Osteopathic Assoc., 56, 345-349.

VOGT, P. K. AND H. RUBEN 1960 Studies on assay and multiplication of avian myelobastosis virus. Virology, 19, 92-104.

VOGT, P. K. AND N. LUYKX 1963 Observations on the surface of cells infected with Rous Sarcoma virus. Virology, 20, 75-87.

von BERTALANFFY, L. AND I. BICKIS 1956 Identification of cytoplasmic basophilia (RNA) by fluorescence microscopy. J. Histochem. Cytochem., 4, 481-493.

WELLER, T. H. AND A. H. COONS 1954 Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. Pros. Soc. Exptl. Biol. Med., 86, 789-794.

WHEELER, C. E., C. M. CANBY, AND E. P. CAWLEY 1957 Long term tissue culture of epithelial-like cells from human skin. J. Invest. Dermat., 29, 383-392.

WILDY, P., M. G. STOKER, I. A. MACPHERSON, AND R. W. HORNE 1960 The fine structure of polyoma virus. Virology, 11, 444-457.

WILE, U. J. AND L. B. KINGERY 1919 The etiology of common warts. Preliminary reports of an experimental study. J. Am. Med. Assoc., 73, 970-973.

WILLIAMS, M. G. 1961 Histochemical observations of Verruca vulgaris. J. Invest. Dermat., 37, 279-282.

WILLIAMS, M. G. AND R. SHEINEN 1961 Cytological studies of mouse embryo cells infected with polyoma virus using acridine orange and fluorescent antibody. Virology, 13, 368-370.

WILLIAMS, M. G., A. F. HOWATSON, AND J. D. ALMEIDA 1961 Morphological characterization of the virus of the human common wart (Verruca Vulgaris). Nature, 189, 895-897.

WOLF, M. F. AND S. B. ARONSON 1961 Growth, fluorescence, and metachromasy of cells cultured in the presence of acridine orange. J. Histochem. Cytochem., 9, 22-29.