AN ELECTRON MICROSCOPIC STUDY OF HUMAN ORAL EPITHELIUM
FROM HERPES SIMPLEX LESIONS

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I INTRODUCTION

Since the pioneering efforts of Leeuwenhoek in the latter part of the seventeenth century with the light microscope, man has made great strides in the recognition of the fine structures of cells and the effect which microorganisms and other environmental agents have upon them (44). Among the many micro-organisms that cause disease in man, viruses play an important part. New techniques and instruments have made it possible to study these viruses more closely and to find out more about their functions.

One of the most common and widespread pathological manifestations of the oral mucosa is that produced by the virus of herpes simplex. Within the last decade, recognition of clinical entities not previously appreciated as manifestations of this virus have become evident; these are the primary and visceral infections of herpes simplex (5). New instruments and techniques for the study of viral agents and the understanding of their behavior have been devised. One of these newer instruments, the electron microscope, has made it possible to study not only the cells affected by viruses, but also the virus itself.

In recent years, the literature reveals that the electron microscope has been used extensively to study the cells of herpetic simplex lesions and also the intranuclear inclusion bodies found in these cells. Most of this work
has been done on animals or chick embryos that have been infected with the herpes simplex virus, but nothing was discovered in the literature describing the changes found in human epithelial cells of the herpetic simplex lesion.

Before the electron microscope could be used successfully for tissue investigation, many obstacles dealing with the methods and materials to be used in obtaining specimens and their preparation, fixation, embedding and sectioning, had to be overcome. Gradually these problems were conquered and a new field was opened, because the resolving power of this instrument is greater than that of any other instrument previously available.
This study was undertaken for a twofold purpose. The first purpose was to study the changes found in human oral epithelial cells of herpetic simplex lesions, using the electron microscope. These cells were then to be compared with the electron microscopic findings of normal human epithelium and cells in herpetic simplex lesions described in animal experimentation.

The second phase of the experiment was to describe a method by which human oral soft tissues can be prepared and studied with the electron microscope.
A. Herpes Simplex

In 1919, a man by the name of Lowenstein gave the first published report that the virus from a human herpes simplex lesion would produce specific lesions on the cornea of the rabbit. Since that time, great strides have been made in the study of this disease.

The virus of herpes simplex is one of the most common infectious agents of man. The clinical conditions known to be caused by this agent can be classified under diseases of the skin, mucous membrane, eye, central nervous system and miscellaneous areas.

These diseases have many things in common: (1) The tissues attacked are usually those of embryonic ectodermal layers; (2) Vesicle formation in the epithelial layers is a visible manifestation; (3) Intraneuronal inclusion bodies are histologically characteristic; and (4) The virus can be isolated with relative ease from infected tissue (20, 40).

In 1930 Andrewes and Carmichael (2) observed that a large proportion of the adult population had neutralizing antibodies against herpes virus in their blood. They also found that people with the antibodies had recurrent attacks of the herpes simplex, and those who did not have antibodies had no disease. These findings did not follow the usual pattern of infectious diseases and since herpes did not seem to spread from patient to patient but seemed
to be provoked by some nonspecific stimulus, it was suggested that herpes was not an infectious agent but was endogenously generated in the human organism (14). Later on, in 1938, Dodd and co-workers (13) solved some of the mysteries surrounding herpes simplex. They routinely isolated the herpes virus from the mouths of children suffering from a common form of stomatitis. These children also developed neutralizing antibodies to the virus upon recovering from the stomatitis (9). This showed that upon first contact of certain persons with herpes virus, a typical infectious disease resulted and the virus behaved like other infectious agents.

Terminology has caused much confusion when describing the disease caused by herpes simplex virus. Aphthous stomatitis, ulcerative stomatitis, herpetic stomatitis, recurrent habitual aphthae, primary herpetic gingivostomatitis, recurrent herpetic stomatitis, trench mouth, Vincents, canker sores, fever blisters, dyspeptic ulcers, were a few of the names given to this disease entity (5, 20, 38, 49). The isolation of herpes simplex virus from the mouth in this disease and the discovery of specific antibodies to herpes simplex virus have proven beyond a doubt the cause of this disease (2, 9, 15). Now the manifestations of herpes simplex infections can be divided into two major groups: (1) Those that are primary, occurring at the onset in a patient without antibodies in the blood, and, (2) Those that are recurrent, occurring in a patient with specific herpes simplex anti-
bodies in the blood (5, 20, 38, 40, 49). Therefore, the disease can now be named either primary herpes or recurrent herpes, with the area involved added to these two terms for clarity.

1. Clinical course

The clinical picture of herpes simplex infection of the oral cavity is not always a simple one. Other systemic or local conditions may precede, be superimposed upon, or follow the actual herpes simplex infection. The primary infection in a non-immune host with the herpes virus results in either of two different entities: inapparent sub-clinical infection, or clinical disease. Initial infection in most humans is asymptomatic. In probably less than one percent of the population, primary infection produces recognizable clinical disease (5, 42).

The primary attack of the virus usually occurs in childhood between the ages of two and five (13, 20, 38, 41, 49). However, primary herpes infection may occur on the skin, vulva, or eye and may be delayed until adult life (38). Once an individual has contacted the virus, lifelong antibodies will be formed (2, 9, 40). The antibodies are found in most older children and adults, but not in most younger children. Recent work by Ellison et al. (15) has shown that antibodies of herpes simplex are regularly transmitted across the human placenta in significant amounts to give infants a passive immunity.

The primary infection of herpes simplex virus may produce painful oral symptoms that have an acute onset.
They are associated with lymphadenopathy, high fever and malaise, giving the appearance of an acute, serious, systemic disease. Although the clinical course is continuous, it may be divided into three stages: (1) onset, (2) acute stage, and (3) recovery. The onset is characterized by loss of appetite, headache, malaise, a burning itching sensation in the oral cavity, a metallic taste and dryness (3). The acute stage demonstrates red, swollen, tender gingiva and oral mucosa without necrosis of the gingival papilla (49). There may be a rise in body temperature to about 103 degrees F. accompanied by pain. Tiny grape-like blisters may form and be filled with a clear fluid. These vesicles rupture, leaving an ulcer which becomes secondarily infected. The ulcer becomes covered with a pseudomembrane, grayish yellow in color. The margins of the ulcer are a bright red (3, 5, 42, 49). The ulcers can become widespread and involve any part of the oral mucosa, including the tongue and tonsils. Profuse salivation is another characteristic of this stage. The recovery stage begins after the vesicles rupture. Healing proceeds slowly. Granulation tissue forms on the ulcers and then an epithelial covering is formed. The whole process covers a period of about fourteen days. The primary infection of herpes simplex is very contagious and must not be confused with the non-contagious necrotizing ulcerative gingivitis (49). The best known form of herpes simplex disease is the recurrent herpetic lesion. Once an individual has contracted the primary
infection of the herpes simplex virus, the secondary stage, or recurrent herpes lesion may occur at any future time. It seems that the virus lies dormant in the tissue at the primary site of infection until stimulated into activity (3, 5, 18, 49). Although the primary infection is not usually seen clinically, the recurrent or secondary stage is manifested clinically in almost everyone who has had the primary infection (20). The lesions occur particularly around the mouth, nose, lips and oral mucosa. They also can be found on the penis, in the urethra and most any other place on the skin.

Clinically, small clear vesicles form on an erythematous base. Local itching and burning occur as they appear. In the mouth, the vesicles soon rupture, leaving ulcers which become secondarily infected. These lesions are similar to those which occur in the primary form and heal in about fourteen days. These lesions can occur one at a time, or in groups (5, 49). Other clinical signs and symptoms such as high fever, nausea, malaise, loss of appetite, excessive salivation, usually are not seen in the recurrent form of the disease.

2. Etiology

The determining etiological factor is the herpes simplex virus, transmitted by saliva, direct contact or other routes of inoculation (3, 16). Sex, diet, climate and genetic factors do not seem to have a decided influence. A lowered general resistance, metabolic changes, upper respiratory infections and certain febrile diseases, have been suspected as predisposing factors (3).
Kerr (20) stated that trauma, sunlight, menstruation, colds, allergies and elevated body temperature are the most prominent secondary or predisposing factors to herpes simplex disease. Thoma and Robinson (49) include food sensitivity and emotional or psychic changes as other trigger mechanisms. Burkett (8) lists pregnancy, hyperthyroidism, leukemia and nutritional deficiencies as predisposing factors. In recent years, lysogeny has been given attention as being an answer to the persistence of herpes simplex in dividing epithelial cells between recurrent attacks (5).

3. Diagnosis

Obtaining a complete history from the patient as to the course and symptoms of the condition, together with clinical findings usually will provide enough evidence to make a correct diagnosis of herpes simplex infection. Tests such as smears are now available that will reveal the virus. Antibody tests can be made as infected persons have antibodies formed at the onset and during convalescence.

Differential diagnosis should be made to eliminate acute ulcerative gingivitis, erythema multiforme, primary and secondary syphilis, pemphigus, and periadenitis mucosa necrotica recurrens or chronic recurrent aphthae.

The latter condition is very often confused with herpes simplex infection. According to Thoma and Robinson (49), the disease runs a course of six weeks. The lesion is not herpetic and the cause is not known. The lesion differs from the herpetic type by its raised borders and long course. It leaves scars at the area of occurrence.
Ruchman and Dodd (39) say that periadenitis mucosa necrotica recurrens occurs on gland bearing mucosal surfaces. The lesion forms a plaque with a swelling and burning sensation. No virus has been found associated with the condition. Work done by Rankow (37) reveals that no virus is associated with this disease. He suggests that nervous tension and fatigue may be a cause. This has not been proved.

Stark (48) studied the antibody titer of patients with chronic recurrent aphthae and found those without a history of herpes simplex infection did not have a titer to the herpes virus.

Sobel and Eller (45) call the condition Behet's syndrome or aphthosis. Dense inflammatory reactions consisting of leucocytes, round cells, and wandering cells are characteristic.

4. Histopathology

Within a few hours after the cutaneous or mucosal epithelial cells are infected with the herpes simplex virus, the cytoplasm becomes edematous and the cells become enlarged, producing ballooning characteristics (5). Marked intercellular edema also occurs and forms a vesicle. The vesicle is a result of fluid accumulation which is confined by the stratum corneum. At first only the prickle cells are involved, but later all the epithelial cells in the vesicle become involved, down to the connective tissue. Polymorphonuclear leucocytes appear in the corneum and later in the vesicle as the lesion progresses (19).
Multinucleated giant cells can be found in the base of an early lesion. These can be found in smears as well as in histologic sections (5, 19). The changes that occur in the nucleus of infected cells are the most important histologically. It is in the nucleus that the virus grows and develops in the form of inclusion bodies. The inclusion body is an organized structure found in the cytoplasm or nucleus of cells. Inclusion bodies are found in the nucleus of epithelial cells in herpes simplex infection, and fill the entire nucleus. Some observers have concluded that the inclusions represent a stage in viral multiplication and are composed, at least in part, of viral material (5, 11, 12, 16, 28, 40). Others have inferred that they consist solely of abnormal products of cellular degeneration or metabolism (1).

Originally, Lipschütz (5, 25, 38) described a body as the typical herpes simplex inclusion or "type A" inclusion body. This body characteristically appeared in the nucleus of cells of tissue infected by the herpes simplex virus. These inclusions were considered aggregates of viruses in the nucleus of the cell. The inclusion body was described as a round eosinophilic body found in the center of the nucleus. The Lipschütz body is now considered to be the end stage of a dynamic process of viral development.

In recent years cytochemical studies and electron microscopic studies of these inclusion bodies have revealed many new characteristics not seen before. It is
now believed that the virus itself does not enter the cell, only the nucleic acid. The virus loses its infectivity for a while after entering a cell and this is called the eclipse or dark period (5). Cytochemical studies done by Crouse et al. (12) on the intranuclear inclusions of herpes simplex indicate that the formation is a dynamic process involving many stages. In early inclusions, nucleoprotein of the desoxyribose type is present. In the older "type A" inclusions the nucleoprotein is gone, leaving a residue.

Morgan et al. (25, 26, 27), on studies with the electron microscope of infected chorioallantoic membranes, found the herpes simplex virus developed in the following manner. First there was a margination of the fine dense reticular material of the infected nuclei. Small dense primary or central bodies, 30-40 millimicrons in diameter, were formed. A membrane, 6-8 millimicrons in thickness and 70-100 millimicrons in diameter, formed and enclosed the central body. The nuclear membrane would disrupt at any stage of infection and liberate the virus into the cytoplasm. A second membrane was then formed, having a diameter of 120-130 millimicrons in the cytoplasm. This body then was released into the extracellular fluid. The mitochondria showed swelling and vacuolization, but no apparent connection with the virus.

Coriell et al. (11) did electron microscopic studies of the elementary bodies from the vesicle fluid of herpetic lesions. Characteristic morphology was shown. The particles varied from spheres to daughter shaped forms with
clear centers. No particles were present in lesions 48 hours old. It is assumed these changes are either from enzymatic lysis or from osmotic pressure changes.

Munk et al. (28) showed a particle which was round in shape and 116 millimicrons in diameter. These particles were not homogenous with regard to electron resorption, indicating that the virus is composed of a central structure surrounded by an area of lower density. These lesions were over 48 hours old.

Farmer (16) isolated viruses from herpetic lesions and transplanted them to the chorioallantoic membrane of fertilized hens eggs. The lesions and virus formed on the membrane were identified. Biopsy showed intranuclear inclusions in the epithelial cells adjacent to the lesion of eosinophilic type. These lesions were over 24 hours old.

Scott et al. (41) studied the growth of herpes simplex viruses in rabbit corneal cells in tissue culture. Biopsies were made every two hours after infection. The first two to six hours showed a loss of nucleoli, and the chromatin of the nucleus changed from a normal fine network to a granular structure. Masses of condensed chromatin formed irregular strands while the background remained granular. In the six to ten hour studies a pinkish material appeared, scattered throughout the nucleus. This material coalesced into two to three larger masses separated by chromatin fibers and finally into one large mass filling the nucleus and pushing the chromatin to the sides of the nuclear membrane. Cells in this stage also showed
changes in the first stage. Twelve hours showed cells in all stages of inclusion development. No cytoplasm changes were noted. The virus seems to disappear during the first six hours after infection and reappears when the inclusion forms (43). The so-called characteristic inclusion body of herpes simplex represents only one change and this is the last change.

It is apparent that no single stage of this dynamic process can be considered the typical inclusion body, for changes found at any one time will depend upon the number of infected cells and the duration of the infection. The nucleus will be filled with a basophilic inclusion material in the beginning, and at the end will have a small eosinophilic inclusion body.

No information is yet available concerning the stage of viral development at which the attribute of infectivity is acquired. Some thought is being given that the viral particles are not infective until they obtain the second membrane (26).

B. Electron microscopy

For two-dimensional structural analysis, it was shown that it is impossible to extend the resolving power of an optical microscope below one-half the wave length of the illuminating light so that 0.1 micron is the limiting resolution afforded by any optical microscope. It was therefore necessary to find some other means to obtain better resolution. No progress was made until the wave properties of electrons were revealed by De Broglie (44)
in 1924. Two years later, Busch (10) showed that the magnetic or electrostatic fields possessing rotational symmetry act as true lenses for an electron beam travelling approximately along their axis of symmetry. Physicists found it was now possible to design a microscope with a beam of electrons as an illuminating system and with magnetic or electrostatic fields as lenses (21). From this time, development of a high resolving power electron microscope was relatively rapid. In 1935 the first electronic image having a resolving power greater than that obtainable with the light microscope was developed.

The electron optical theory shows that it is possible to build electron optical counterparts of most optical instruments. The electron microscope is in theory a rather exact counterpart of the light microscope. Because of the necessity of using high accelerating potentials in the production of the electron beam and of handling the beam entirely in a vacuum, its physical form is completely different, and it imposes quite different requirements for specimen preparation (44).

All specimens must be placed in the vacuum under which the instrument is operated so that there is no possibility of observing wet or living specimens. The specimens must also be so thin as not to show appreciable electron absorption, because image contrast arises primarily from the absence of electrons scattered out of the direction of the beam and not from the absence of those absorbed in the specimen. Electron absorption also causes
over heating of the specimen and rupture.

Since electrons of one velocity are used, atomic densities of the subject are the primary cause of variations of intensities at the image. Specimen preparation has the following requirements: (1) They must be thoroughly dehydrated and fixed prior to examination; (2) Adequate contrasts of atomic density must be present or be induced in the specimen; (3) Specimens must be no thicker than 0.2 microns if resolutions less than 0.1 microns are desired, and even less for optimum resolutions (44).

It has become possible to examine cellular structures at electron microscopic magnifications under the influence of various fixatives (35, 36). All fixatives tried except osmic acid, caused a gross contraction during drying which was accompanied by submicroscopic distortions. No mitochondria are visible after formalin fixation. Other investigators have come to the conclusion that fixation in osmic acid most closely preserves the living cell structure (4, 7, 31, 32).

Tetroxide is the highest known oxide of osmium and is produced by the oxidation of osmium in aqua regia. Its value in fixing is dependent upon its ability to act as a strong oxidizing agent and to affect various components of living material differently, as determined by their composition and reactivity. Little is known of the reaction of osmium tetroxide with proteins, but in preliminary studies it has been shown to produce an initial gelation of protein solutions. It is postulated that by
virtue of its tetrahedral configuration, osmium tetroxide may form polymer-like structures with proteins. This initial gelation may then be followed by a further oxidation and the production of soluble end products, which after long fixation wash out of the cell (36).

The size and shape of cytoplasmic components are successfully studied in smeared and cultured cells but the nucleus and perinuclear regions are too electron-opaque for observation. To study these regions it has been necessary to cut sections from whole tissue 0.05 to 0.20 microns thick. To accomplish cutting sections that thin it was necessary to develop new microtomes as the old conventional ones were not geared for ultrathin sectioning. Many different types of microtomes were made and not many proved too efficient. The earliest microtome built especially for electron microscopy was based upon the principle that the thickness of sections may be determined by the rate at which the objective moves and the speed of a rotating blade (17, 30). This microtome has been abandoned in favor of newer and more effective designs.

It has recently been found to be extremely important, for the sectioning of plastic embedded tissue, to avoid contact between the block and the cutting edge during a noncutting stroke. Methacrylate undergoes a slight expansion after the cutting blade has passed through it, so that if the block touches the knife on the upstroke, undesirable wetting and dulling of the blade occur (44). Most workers are now concentrating on new designs which
avoid block and knife contact on the non-cutting stroke. The Servall "Porter-Blum" microtome accomplishes the non-contact stroke and the specimen advances continuously by rotating in front of a stationary knife. This instrument is excellent for cutting plastic sections 0.5 to 0.025 microns thick. Once the mechanical advance has been achieved the quality of the embedding material and the cutting edge are the limiting factors in the achievement of satisfactory sections.

A plastic embedding was introduced by Newman et al. (29) which is now in general use. Tissues are immersed in liquid n-butyl methacrylate and incubated at 45 degrees C. with a catalyst or under ultraviolet light (24). This polymerizes and fixes the tissues in an extremely hard, colorless resin. The hardness of the embedding may be controlled by the amount of catalyst used or by the addition of methyl methacrylate. This embedding has the advantage of being as hard as desired and thus eliminated the great compression inevitable in sections of celloidin-paraffin embedded material. Its disadvantages are introducing occasional gross injuries to tissue during polymerization, needing a supporting film under the electron beam and undergoing expansion during sectioning. However, the thinnest sections and therefore the finest resolutions have been achieved using this embedding (46, 47).

Since the increased magnification with which tissue sections are observed, increasingly smaller knife defects become evident and destructive of the sections. With the
advent of ultrathin sectioning, it was found necessary to replace stropping of steel knives or razor blades with fine abrasive powder. This is a very expensive, time consuming and not always successful method (6, 46). This brought about the development of the glass knife (23). The glass cutting edge is superior to a commercially sharpened steel knife, is low in cost, relatively easy to make, and therefore available to all.

The electron microscope has made it possible to follow the development of viral and nonviral cellular components with the passage and growth of the tumor, therefore further elucidating the proliferate mechanism of these cells (44).
IV EXPERIMENTAL METHOD

BIOPSY

Excision biopsies were obtained from six patients attending the Ohio State University Dental Clinic for treatment. Five biopsies were made on patients with herpetic simplex lesions and one biopsy was made on normal tissue. All of these specimens were taken from the oral mucosa. The patients ranged in age from twenty-five to forty years.

The five herpetic simplex specimens were taken from the following areas of the oral mucosa; two from the mucobuccal fold of the lower arch, one from the palate in the second molar region, and two from the buccal mucosa of the lower lip. The normal tissue was obtained from the buccal mucosa of the lower lip. The normal tissue presented the usual pink color characteristic of healthy mucous membrane.

The surface of the area to be removed was prepared with a topical anesthetic. Xylocaine hydrochloride was injected locally around the area to be excised.

The site which was chosen to be removed was outlined to include only the lesion, limiting the amount of surrounding tissue to a bare minimum. The depth of the incision was also limited to include only epithelium and as little connective tissue as possible. Sharp, disposable types of scalpel blades were used along with tissue forceps. The operative site was sutured and the patient dismissed for four days, after which the silk sutures were removed.
**FIXATION**

The method used for fixing the tissue was a modification of that described by Palade (32). Immediately after taking the biopsy the specimen was placed in a three inch Petri dish containing one percent osmium tetroxide fixative, buffered at a PH of 7.3 to 7.5 with sodium acetate-sodium vernal. The specimen was then cut into 1 mm. squares with a razor blade which allowed the fixative to penetrate the tissue thoroughly and quickly. Care had to be exercised while cutting the specimens into small squares as fumes from the osmium tetroxide fixative will fix living tissue of the operator if they come in contact. To accomplish this phase of the procedure safely, the cutting was done under a hood.

The specimens were left in the fixative for fifteen to twenty minutes at room temperature then run through a series of graded ethyl alcohol baths to dehydrate the tissue. The series consists of fifteen minutes each in 50 percent, 75 percent, 90 percent and 100 percent ethyl alcohol.

**EMBEDDING**

After dehydrating the tissue in the last fifteen minute bath of 100 percent ethyl alcohol, the specimen is prepared for embedding. The method used was a modification of that introduced by Newman et al. (29). The tissues were immersed in a liquid combination of n-butyl methacrylate (20 ml), methyl methacrylate (5 ml) and 0.25 grams luberco, a catalyst. The latter two components control the
hardness of the acrylic. This mixture was diluted half and half with 100 percent ethyl alcohol and the specimen was left for fifteen minutes in the solution. The specimens were then put through three fifteen minute baths of n-butyl and methyl methacrylate without the alcohol. This insures a complete penetration of the methacrylate into the tissue specimen.

The next step in the embedding process is to fill several #4 gelatin pill capsules with the clear methacrylate liquid and place one piece of tissue in each capsule. The tissue will sink to the bottom of the capsule. The top of the gelatin capsule is replaced to prevent evaporation. The capsules with specimens are then placed in an oven at 45 degrees Centigrade for twenty-four hours to harden the acrylic. The gelatin pill capsule acts as a matrix to form a small block of acrylic containing the specimen (Fig. 1). The gelatin capsule is easily removed by soaking in water after the acrylic has hardened.

SECTIONING

One of the most difficult and important steps in the tissue preparation is that of sectioning the tissue. The sections have to be ultrathin for the best resolution. The knives used in this study were made from glass (23). The knives were made by breaking a strip 1 1/2 inches to 2 1/2 inches wide and about 12 inches long from a sheet of plate glass, approximately 3/8 inches thick. The fracture line should be as straight and smooth as possible. A series of
straight parallel scorings at 45 degrees to the long axis were then made on each strip one inch apart and on the opposite surface from the first scoring. The parallelograms (Fig. 1) thus outlined were broken off and examined under a light microscope for a smooth cutting edge. A metal water trough to float the sections off the edge of the knife was attached with dental compound or sticky wax to the top surface of the knife. This trough was sealed tight so as not to leak water (Fig. 1). The knife was then placed in the microtome knife holder at a slight angle (about 5 degrees) to the face of the acrylic block.

A Servall "Porter-Blum" microtome (34) was used for the actual sectioning. This microtome will section tissue 0.025 microns to 0.5 microns in thickness. The acrylic block holding the specimen was placed in the microtome chuck which moved the specimen past the stationary knife, cutting off sections in ribbon form, 0.05 microns in thickness. A section 1½ microns in thickness was cut and placed under the phase microscope to indicate if the proper tissue was being sectioned for study under the electron microscope.

The sections were floated off the knife edge by a 10 percent acetone solution contained in the water trough on the knife. These sections were then transferred in ribbon form from the water trough, by means of a small water paint brush, to a petri dish containing warm 10 percent acetone solution which allowed the section to smooth out after being sectioned.
The sections were picked up from the warm acetone solution by copper grids 1/8 inches in diameter (Fig. 1). These copper grids are 250 mesh per inch and have been coated with ultrathin coats of colloidin and carbon to support the tissue. The grids are placed on top of four or five sections in a ribbon floating in a petri dish. The sections were then in a row in the middle of each grid. The copper grids were allowed to dry for twenty-four hours and then placed in the grid holder (Fig. 1) which was transferred to the electron microscope for viewing and photographs. An RCA type EMU - 2 electron microscope was used in this study (Fig. 2).
The method used for fixing, embedding and sectioning the biopsied tissue gave good results. Several problems had to be overcome, however, before usable sections were obtained. These problems were primarily due to the inexperience of the author in the laboratory procedures necessary for good electron microscopy. The technique for making the glass knives took much time and practice before suitable cutting edges were obtained. Once the technique was acquired the knives were more easily made.

Controlling the hardness of the acrylic embedding material was another variable to overcome. In the beginning many specimens were ruined because the acrylic was too soft and would not section properly. By controlling the amount of catalyst carefully, the proper hardness was obtained. No set formula can be followed to obtain the correct hardness of the acrylic, only trial and error. However, 20 ml. butyl and 5 ml. methyl methacrylate plus 0.25 grams luberoo gave a good result.

During the sectioning procedure careful observation had to be kept on the water level in the trough on the knife blade. Too much water would wet the specimen and the section would not be cut properly. If too little water was used the sections would not float off the edge of the knife after being cut. The position of the specimen to the knife edge and the speed of the cutting were other factors which, if not controlled, would ruin the specimens. Trial and
error will correct these factors and good specimens can be obtained when they are controlled.

When viewing the specimens under the electron microscope, careful control of the intensity of the electron beam and minimal time spent on each specimen had to be observed. Too much intensity would burn the tissue. If the tissue was allowed to remain for too long a period, 1/2 hour or more, under the beam, cellular changes would become evident.

Figures 3, 4, 8, 9, 10 and 11 are photographs of epithelial cells in herpetic lesions. Figures 5 through 7 are of normal epithelial cells. The outstanding characteristic of the herpetic cells is their large size. By comparing figures 8, 10 or 11, with figure 7, all of which have the same magnification, it can be seen that the cells from the herpetic simplex lesion demonstrate ballooning. Figures 3 and 4 show cells enlarged 4,050 times which are larger than normal cells, figures 5 and 6, enlarged 8,100 times. The herpetic cells appear more granular with evidence of hydropic degeneration, figures 3 and 9. The normal cells are more homogenous and the granules are closer together (Figures 5, 6 and 7). Normal and abnormal cells are almost impossible to find on one section because of the small area involved.

The nuclear membrane was found to be very irregular in appearance. Figures 9 and 11 show the irregularities found in the nuclear membrane of the cells studied from the herpetic lesions. All of the nuclear membrane seemed
to be intact. The nuclear membrane observed in the normal cells is smooth in outline. The size of the nucleus itself is also increased in the herpetic lesion cells, but not in proportion to the rest of the cell. This nuclear size increase is evident by comparing figures 3 and 4 with figures 5 and 6.

Although no virus could be demonstrated in the nucleus of the infected cells studied, one finding was present in these cells that could not be found in the normal cells. The presence of intranuclear inclusion bodies was a constant finding as is indicated in figures 8 through 11. These inclusions are round, homogenous bodies surrounded by a clear zone in the nuclear cytoplasm. These bodies varied in size from 80 millimicrons to 900 millimicrons. The position of these bodies in the nucleus are to be found near the periphery and not centrally located. In some of the cells, figures 9 and 10, two inclusion bodies can be seen; in other sections only one inclusion body is present.

The sections studied retained their characteristics through the preparation of the tissues as is evidenced by the presence of the mitochondria and other minute cellular structures as seen to the right of the nuclear membrane in figure 8. The cellular outlines and intercellular bridges can be seen in figures 3 and 4.

The nucleoli of various cells can be seen in some sections and not in others. Figure 9 shows two nucleoli present. The sections which do not show nucleoli, figures 3,
7 and 8, were probably not cut through that part of the cell where the nucleoli would be found. The nucleoli are round in shape and appear rather homogenous. They are darker than the rest of the cell, indicating they possess greater electron scattering properties than other parts of the cell.

Tissue sections cut thicker than 0.1 microns were of little value for study as the resolution was very poor. The thinner the sections, the better the cell structure could be seen. Sections 0.05 microns thick allowed for large magnification without loss of minute detail.
The cytoplasm of the epithelial cells studied were edematous and swollen, producing ballooning characteristics. This agrees with the findings of other workers (5, 40). The nucleus was also enlarged and the nuclear membrane irregular in outline. This is probably due to the degeneration of the cell. The nuclear membrane was very distinct and showed no separations.

Fine, thin, cellular membranes can be seen in figures 3, 4 and 7. Similar cellular membranes were described by Pease (33) in electron microscopic studies of normal epithelium, but Laden (22) states that no cellular membranes were observed in his study on epithelial cells.

The cellular membrane of one cell seems to join with the cell membrane of an adjacent cell and forms spaces with intercellular bridges between the cells (Fig. 3).

Although no distinct membrane can be seen around the nucleolus in figures 4, 9, 10 and 11, their periphery is slightly darker, suggesting the possibility of a membrane.

The intranuclear inclusion bodies found in the cells from the herpetic lesions were not typical of those described by Scott (41, 43) and Morgan (25, 26), but did resemble those described by Munk (28). The cells studied in this work were from lesions 3 to 7 days old. These inclusion bodies would most likely be the last stages of viral development and would be of the eosinophilic or non-infective type inclusion. Further study done on early
lesions still in the vesicle stage might confirm work done on animals and chick embryos showing the dynamic process of herpetic simplex viral development.

The use of osmium tetroxide for the fixation of the biopsied tissue proved to be a fast and efficient method. The tissues were well preserved and retained their minute cellular characteristics. The glass knives used to section the specimens performed nicely, but had to be changed frequently because of dulling. This was of little consequence as new knives are made quickly and inexpensively.

The use of methyl and butyl methacrylate for embedding the specimens to be sectioned worked out satisfactorily. Correct hardness of the material had to be obtained so as to make good sections. The methacrylate makes for easy handling of the specimens as they are small and would prove to be difficult in other media.

The microtome used in this work can cut tissue 0.025 microns in thickness, which will give good resolution. It is easy to operate and is very consistent in producing good sections, provided other factors are constant.
VII CONCLUSIONS

1. The use of osmium tetroxide as a fixative for electron microscopic study of oral epithelium is favorable.

2. Glass knives used to section the specimens produce ultrathin sections of 0.05 millimicrons that are suitable for electron microscopic viewing.

3. Methyl and butyl methacrylate combination produces a good embedding material.

4. The Servall "Porter-Blum" microtome is capable of producing ultrathin sections necessary for good electron microscopy.

5. The epithelial cells from herpes simplex lesions show ballooning which may be followed by degeneration.

6. The nuclear membranes of the herpetic cells are irregular in appearance, as compared to normal epithelium.

7. Intranuclear inclusion bodies were seen in the cells from the herpetic lesions. These inclusions were found to be from 80 to 900 millimicrons in size and were surrounded by a clear zone.

8. No intranuclear inclusion bodies were visible in normal epithelial cells.

9. Normal epithelial cells were smaller than the cells infected by the herpes virus and their nuclei occupied a proportionately greater amount of the intracellular space.

10. Cellular membranes and intracellular spaces were observed in both normal and pathological cells.
Fig. 1. Left to right: Copper grid acrylic block with embedded tissue specimen, glass knife with water trough and grid holder
Fig. 2. R.C.A. Type EMU - 2 electron microscope.
Fig. 3. Epithelial cells from a herpetic lesion. Irregular nuclear membranes and granular nuclear cytoplasm. Cells show "ballooning" which may be followed by degeneration. Mag. 4,050 X.
Fig. 4. Epithelial cell from a herpetic simplex lesion. The cell shows ballooning and the nucleus is irregular in outline. Intracellular spaces and bridges are evident. Mag. 4,050 X.
Fig. 5. Normal epithelium from the buccal mucosa. Nucleus fills most of the cell and has a smooth membrane. Mag. 8,100 X.
Fig. 6. Normal epithelium from the buccal mucosa. Mag. 8,000 X.
Fig. 7. Normal epithelial cell. Same as Figure 6. The nucleus occupies most of the cell and is relatively regular in outline. The nucleus is homogenous in appearance. Mag. 16,200 X.
Fig. 8. Epithelial cell from palatal herpetic lesion. The nuclear membrane is the irregular dark line in the center of the photograph. The nucleus is to the left of the membrane and the cytoplasm is to the right. Note the inclusion body surrounded by a clear zone in the upper right hand corner of the nucleus. Mag. 16,200 X.
Fig. 9. Epithelial cell from buccal mucosal herpetic lesion. Note irregular nuclear inclusion bodies, one near the left nucleolus and the other in the lower right portion of the nucleus. Mag. 12,150 X.
Fig. 10. Epithelial cell from buccal mucosal herpetic lesion. The dark irregular line is the nuclear membrane. The large round dark object in the nucleus is the nucleolus. The two smaller objects surrounded by halos are intranuclear inclusion bodies. Mag. 16,200 X.
Fig. 11. Epithelial cell from the palate. Nucleus covers most of photograph except upper right corner. A large nucleolus is seen to the right. Smaller inclusion body surrounded by clear halo is seen near the left border of the photograph. Black spots are carbon artifacts. Mag. 16,200 X.
LITERATURE REFERENCE


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