# GENERALIZED CYTOMEGALIC INCLUSION DISEASE

## A REVIEW AND A REPORT OF THE ISOLATION OF VIRUS FROM CASES OCCURRING IN JOHANNESBURG

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### GENERAL REVIEW

The inclusions of cytomegalic inclusion disease were first described in 1904 by Jesionek and Krolenenglou,[1] who observed them in the kidneys of a stillborn infant. In the same year, Ribbert[2] reported that he had first observed similar inclusions in the kidneys of a newborn infant in 1881 and had subsequently observed them in the parotid glands of 2 older infants. Since then it has become apparent that this type of inclusion is not infrequently found in the salivary glands of infants and young children. They have been reported to be present in 10 - 32% of infants regardless of the cause of death. They were less frequently found in organs other than the salivary glands, but even so their presence has been noted in from 1 to 2% of routine series of autopsies on infants. Most early workers regarded the inclusions as some form of protozoal parasite and as an incidental finding in infants dying from various other causes. Many of the early cases in which there was generalized distribution of these inclusions were regarded as examples of congenital syphilis.

In 1921 Goodpasture and Talbot[3] observed inclusion cells in the lungs, kidneys and other organs of a child dying of bronchopneumonia at 2 months of age. This infant, the fourth in the family, was markedly anaemic at birth. The mother stated that the second and third children had died at about the same age of a similar condition. Goodpasture and Talbot,[3] in 1921, and Jackson,[4] in 1922, drew attention to the close resemblance of these inclusion cells to the affected cells in salivary-gland disease of guinea-pigs which Jackson had described in 1920, and also recalled that Tyzzer[5] had described intranuclear acidophilic bodies in cases of varicella.

In 1925 van Glahn and Pappenheimer[6] described the characteristic cells in the intestines, lungs, and liver of an adult who had died of ulcerative colitis, liver abscess, and pneumonia. In 1930 Pearson[7] drew attention to the cytoplasmic inclusions which had been noted previously, but were otherwise rather ignored in these cases. In 1932 Farber and Wolbach[8] described these inclusion bodies in the salivary glands of 22 of 183 infants between 2 days and 17 months of age. Of these, 2 had congenital syphilis and 1 had measles, 2 were also siblings, the elder dying of haemorrhagic disease, the younger of erythroblastosis foetalis. Since then the same type of inclusion has been recorded in haemolytic disease of the newborn by Wanstrom[9] in 1933, Andrews and Miller[10] in 1935, Vidari[11] in 1940 and Kinney[12] in 1942, who also found them in 4 children with whooping cough.

*Virus Aetiology*

In 1947 Cappell and MacFarlane[13] described the first 2 cases of the condition to be recognized in Britain. These were 2 infants who had died of haemolytic disease of the newborn. In both, widely distributed intranuclear and cytoplasmic inclusion bodies were found. They described these in detail. A notable feature was the failure to detect the characteristic morphological changes in cells other than those that function as epithelium. In their discussion of the nature of the causal agent, Cappell and MacFarlane noted that the lesions are identical morphologically with those attributed to the action of the salivary-gland virus of rodents. By analogy, they suggested that the widespread lesions of infants are probably caused by dissemination of a human strain of salivary-gland virus. They noted further that, although their 2 cases were diagnosed as haemolytic disease of the newborn, iso-immunization of the mother by any of the known blood-group antigens carried by the foetus had been excluded in both cases.

In 1950, Wyatt and his colleagues[14] reported a study of 6 cases of inclusion disease and fully reviewed the findings in previously reported cases. They concluded that the aetiological agent of the disease is a specific virus which *per se* is a common cause of foetal and infantile death, and that the morphology and cytology of the inclusion-bearing cells is pathognomonic of the disease. They also noted that the clinical and pathological pictures are variable, depending on the intensity of viral activity in different organs and tissues. In uncomplicated cases death may result from viral pneumonia, viral nephrosis, viral hepatitis, viral enteritis, and presumably from other mechanisms. In 2 patients viral hepatitis caused cirrhosis of the liver.

In one patient with a clinical picture of diarrhoea, ulcerative enteritis associated with characteristic inclusions was found, and in another the alterations were characteristic of a viral encephalitis. Since the infection occurs during intra-uterine life, it was suggested that it may be an unrecognizable cause of foetal abnormalities and early foetal death. Exfoliative cytological studies of the urine were suggested as a possible method of antemortem diagnosis, because of the frequent massive desquamation of inclusion-bearing cells into the renal tubules. Wyatt *et al.*[14] pleaded for an investigation of this important disease by modern virological methods.

In December of the same year (1950), another comprehensive review by Margaret Smith and Vellios[15] appeared. They, too, noted that the human disease had not been transmitted to experimental animals, nor had a virus been demonstrated by the inoculation of embryonated eggs or tissue cultures. However, they considered that the intranuclear and cytoplasmic inclusions peculiar to this disease were comparable to those associated with known viral infections of man and lower animals, and suggested that the virus concerned was the salivary-gland virus.

They presented 20 cases of inclusion disease of infants and children. In 3 instances the disease was observed in infants dying in the neonatal period. In addition, they reviewed the findings in the previously reported 69 cases, and noted that generalized salivary-gland virus infections occurred in the majority of instances during the first 2 years of life. The infection may occur *in utero* without the mother showing any evidence of infection. Death from the uterine infection may occur *in utero* or in the neonatal period.

In infants under 2 months of age, the most frequent clinical and anatomical manifestations are those of a blood dyscrasia or of hepatic damage. However, the character of the disease may vary, since the viral infection may involve with variable intensity many organs of the body, including the liver, lungs, kidneys, brain and intestines.

In children over 2 months of age, inclusion disease was associated with diverse clinical histories and anatomic changes. At times it may be the principal disease, more often it is associated with another primary disease and plays only a minor role in the child's illness. Smith and Vellios noted, but left unexplained, the associations of pertussis and generalized salivary-gland virus infection in approximately one-third of the cases, including their own series.

The disease has been reported in 5 stillborn infants, and was presumably a major factor in the stillbirth. Approximately three quarters of the 89 cases analysed by Smith and Vellios involved infants 2 years old or younger. Only 1 child was over 5 years of age. Twenty-one infants died within the neonatal period of 1 month, 9 died on the first day of life, and 8 others within the first 2 weeks. In their own series of 1,411 autopsies of infants and children at St. Louis Children's Hospital, they found 15 cases, an incidence of slightly over 1%. Wyatt and his associates noted an incidence of 1·1% in autopsies of 461 infants and children from other St. Louis hospitals.

Only 3 cases of generalized inclusion disease in adults have been reported. In addition 6 adult cases of local lesions, including ulcers of the oesophagus and stomach, and a granuloma of the anus, have been described.

*Diagnosis during Life*

Following up the suggestion of Wyatt and his associates that it might be possible to make a clinical diagnosis in inclusion disease by the identification of typical inclusion-bearing cells in cytological preparations of urine, Fetterman[16] reported the finding of typical inclusion-bearing cells in the urine of a 2-day-old premature infant. This infant was admitted to hospital presenting with jaundice, purpura, hepatomegaly and splenomegaly. There was no Rh incompatibility, the Coombs'

test was negative and serological tests for syphilis were negative. X-ray films of the skull revealed cerebral calcification outlining a portion of the ventricular system. Examination of smears made from a specimen of urine revealed several inclusion-containing cells. A typical example of these showed a large intranuclear inclusion in an enormously hypertrophied cell. The infant died at 4 days of age of generalized inclusion disease. Typical inclusions were found in sections of brain, pituitary gland, thyroid gland, lung, liver, pancreas, and kidneys, thus corroborating the diagnostic value of the finding of the cells in the urine.

Margileth,[17] in 1955, reported the fourth case of cytomegalic inclusion disease up to that time which had been diagnosed before death, the diagnosis being made by the finding of cytomegalic inclusion cells in the urine. This patient was a premature infant weighing 5 lb. 4 oz. at birth. The infant had a fair cry and respirations were established within 1 minute, but it was noted to have many areas of purpura, haemorrhagic macules and petechiae over the face, extremities, and trunk. The placenta and amniotic membranes were normal. Within 12 hours of birth definite icterus had developed. The liver was palpable 1 cm. below the costal margin, and the spleen was 2 cm. below the costal margin. Otherwise the baby appeared to be normal.

This baby's case was followed for 15 months. A moderate haemolytic anaemia developed during the first 2 weeks of life. At 15 days of age the patient had a severe cerebral haemorrhage which was almost fatal. Treatment with cortisone, 5 days of oral cortisone followed by intramuscular cortisone in a dosage of 10 mg. every 12 hours, resulted in dramatic improvement.

### THE VIRUS OF CYTOMEGALIC INCLUSION DISEASE

Cytomegalic inclusions have been observed in the tissues of animals of a number of species. In each case the virus has proved to be species-specific. The salivary-gland virus of rodents is of low virulence. Usually, no apparent disease is produced in rodents inoculated with the homologous virus, but inclusions are found in the salivary gland and in the immediate site of inoculation. However, by intracranial inoculation in young animals, fatal disease can often be produced. The infection can be transmitted indefinitely in series, and the infective agent has the properties of a virus, which it undoubtedly is. In 1954 Margaret Smith[18] reported the successful culture of the virus of salivary-gland disease of mice. She observed cytological changes, including large intranuclear inclusions, in cultures of mouse tissue inoculated with a suspension prepared from salivary glands of mice infected with the virus. These changes also occurred in 2 serial subcultures. After 3 serial passages of the agent in cultures, the disease was reproduced in mice by intraperitoneal inoculation of the supernatant fluid from the cultures. Titration of virus in the cultures showed a significant increase.

The successful culture of the virus of human cytomegalic inclusion disease was then reported in rapid succession, but independently, by Rowe and his associates,[19] by Margaret Smith,[20] and by Weller and his associates.[21]

Rowe and his associates[19] reported the isolation of 3 strains of a virus which produced intranuclear inclusion bodies from spontaneously degenerating cultures of human adenoids. One strain was studied in detail and appeared to be closely related to, or identical with, viruses isolated in other laboratories from a human salivary gland and a patient with cytomegalic inclusion disease.

The adenoids were obtained from children undergoing tonsillectomy-adenoidectomy at the Children's Hospital, Washington, and the Clinical Center of the National Institutes of Health. The 3 strains were recovered, respectively, from adenoids taken from an 11-year-old boy, a 7-year-old girl, and a 6-year-old girl. From each of these patients, after an incubation period of 22 - 51 days in culture, an adenovirus was isolated. From each, after an incubation period of 71, 64 and 34 days in culture respectively, 1 culture in each set showed focal areas of degeneration in the fibroblasts. The changes consisted of sharply demarcated, smoothly outlined, oval or round cells. As the foci enlarged, the central cells became necrotic and densely pigmented, eventually leaving a granular mass surrounded by a thin border of rounded cells.

After the fourth passage the incubation period shortened markedly and the virus passed well subsequently. Cytopathogenic changes were produced in all types of human fibroblast cultures tested, including tonsils, adenoids, foreskin, and embryo skin-muscle. Preparations stained with haematoxylin and eosin showed that numerous cells in the foci contained large eosinophil intranuclear inclusion bodies with margination of chromatin and halo formation. In cultures containing epithelium and fibroblasts, the epithelium remained unaffected, except in rare instances when cells immediately adjacent to affected fibroblasts underwent clumping and inclusion-body formation.

Cytopathogenic changes were not induced in cultures of HeLa cells, KB cells, monkey kidney, or rabbit trachea. Adult and suckling mice injected intracerebrally and intraperitoneally, and rabbits inoculated intradermally and onto the scarified cornea, showed no sign of illness. Infectivity was destroyed by exposure to 20% diethyl ether for 2 hours. The virus did not withstand storage for a week at 4°C. and much virus was lost after quick freezing and storage at −20°C. Slow freezing with storage in dry ice appeared more satisfactory for virus storage. Tests to detect complement-fixing and neutralizing antibodies were carried out, and these revealed that antibodies were present in a high proportion of normal sera.

At the same time, Margaret Smith[20] reported on the isolation of a cytopathogenic virus inducing large intranuclear inclusions, like those occurring in salivary-gland virus disease, from each of 2 infants. In both, the virus had been propagated serially in cultures of human fibroblasts derived from uterine tissue. She concluded that the distinctive cytopathogenic effect and the apparent species-specificity, together with the isolation of each of the viruses from human tissue which contained the characteristic inclusions of salivary-gland virus disease, was evidence that these viruses were strains of cytomegalic inclusion disease.

Finally, Weller and his associates,[21] who had previously reported briefly on the isolation of the agents, described in detail their studies of the viruses isolated from 3 infants during life.

*Viruses Isolated during Life*

The first patient, admitted to hospital when 3 months old, presented with jaundice, hepatosplenomegaly, periventricular cerebral calcification, bilateral optic atrophy, and 1 area of chorioretinitis. The urine contained a few white cells, but no inclusion bodies were demonstrated. A liver biopsy was performed and a 20% suspension in physiological saline was prepared and inoculated in 0·2 ml. amounts into cultures of human embryonic skin-muscle tissue. A cytopathogenic agent was recovered. Histopathologic examination of the liver biopsy showed diffuse alteration of architecture, with extensive bile

stasis, many degenerate hepatic cells, and scattered multinucleate giant cells. There were focal areas of erythropoiesis and numerous mononuclear inflammatory cells in the portal area. Few large cells of cytomegalic type were found. Eight months later hepatosplenomegaly was still present, but the infant appeared well nourished.

In the second case, petechiae, jaundice and hepatosplenomegaly were noted 8 hours after birth. There was no evidence of Rh or major blood-group incompatibility. In films made from the urine on the 5th and 8th days of life, large epithelial cells, containing multiple, round, homogeneous, brightly eosinophilic, cytoplasmic inclusions, were observed. No intranuclear inclusions were seen.

A liver biopsy was taken on the 25th day of life. Foreskin cultures inoculated with a 10% suspension prepared from the liver biopsy, developed focal degeneration. Histological examination showed preservation of lobular pattern, but loss of cell-cord pattern with formation of many large multinucleate giant cells. Rare foci of liver-cell necrobiosis, and a diffuse inflammatory reaction, consisting of mononuclear cells, eosinophils, and polymorphonuclear cells, were present throughout the parenchyma and in the portal tracts. No cells with intranuclear inclusions were seen. The picture was identical with that of neonatal hepatitis described by Craig and Landing.

Two foreskin cultures inoculated with urine collected on the 36th day of life developed characteristic cytopathic changes. At the age of 2 months this baby appeared normal except for the persistence of hepatomegaly, yet virus was again recovered from urine collected on the 91st day of life.

The third patient, a baby, was found to have hepatosplenomegaly and periventricular cerebral calcification at the age of 6 weeks. Subsequently progressive chorioretinitis developed. Cytomegalic inclusion cells were demonstrated in the urine on several occasions, and specific changes developed in foreskin cultures inoculated with a specimen of urine forwarded in melting ice.

In a study of the cytopathic range of the virus isolated from the first patient, it was found that focal or generalized cytopathic changes developed in fibroblasts derived from human foreskin, from human embryonic skin muscle or lung tissue, and in a cultured line of fibroblasts. Similar changes occurred in cultures of human cells and in the outgrowth from human testicular tissue. The inoculation of cultures of human epithelial cells of various types was not immediately followed by the development of overt cytopathic changes. In stained preparations changes could be demonstrated in some instances. The marginal cells of plaques of squamous epithelium, in contact with affected fibroblasts, often showed changes with intranuclear inclusions.

No evidence of growth was seen in cultures of mouse and of chick embryonic tissue, or in cultures of monkey kidney and rabbit testis. No evidence of illness was produced in rabbits after intravenous inoculation, or in newborn mice after intracerebral and intraperitoneal inoculation.

Heating a cell-free suspension of virus for 30 minutes at 56°C. resulted in complete loss of infectivity. Frozen at −50°C., infectivity appeared to be well maintained.

Examination of stained preparations revealed a marked alteration of nuclear structures. The earliest change consisted of the presence in the nucleus of 1 - 4 minute granular bodies staining amphophilic or weakly eosinophilic. In nuclei in which slightly larger inclusions were present there was loosening and coarsening of the chromatin reticulum. Some of the larger inclusions appeared to be composed of masses of small particles of this nature. In the paranuclear position there was an oval zone where the cytoplasm stained more eosinophilic and appeared to be composed of a mass of fine granules. Infected cells, apparently about to undergo dissolution, were often noted to contain one or several large, round, amphophilic to eosinophilic cytoplasmic inclusions.

These viruses differed from the herpes virus in that they did not induce the rapidly spreading cytopathic changes observed with herpes virus and also failed to produce illness in newborn mice. Furthermore, antisera from patients convalescent from herpes simplex infection failed to neutralize the strain of virus isolated from the first case (Davis). The foci of cytopathogenic change produced by these agents are more prominent than those seen with varicella virus. No neutralizing antibody to the Davis strain was found in 6 sera known to contain varicella antibody. These agents are apparently related to those isolated by Smith and by Rowe and his co-workers. It also appears that these agents are common associates of man.

Weller concluded that the aetiological import of the isolation of these agents during life cannot be defined at present.

### STUDIES IN SOUTH AFRICA

About 2 years ago, a systematic study to define the incidence and importance of cytomegalic inclusion disease in this region was undertaken in collaboration with the staff of the Transvaal Memorial Hospital for Children. A watch was kept by one of us (I.K.) for possible cases of cytomegalic inclusion disease among the newborn babies and the infants under 2 years of age admitted to the hospital. In particular, infants presenting with jaundice or other signs of blood dyscrasia and enlarged liver and spleen were suspected of being sufferers from this condition. These patients were subjected to the usual detailed clinical and clinico-pathological examination. In addition, urine was collected under suitable conditions for special study for evidence of infection with the cytomegalic inclusion disease virus. A part of the sample was preserved by the addition of 1 ml. of 10% formalin to 9 ml. of urine. The rest was sent untreated to the laboratories of the Poliomyelitis Research Foundation for an attempt at the isolation of virus. It was stored at −20°C. until this could be done.

#### *Direct-examination Methods*

The sample preserved with formalin was centrifuged and films on clean glass slides were prepared from the deposit for microscopic examination. These films were fixed for 20 minutes by immersion of the glass slides in a mixture of equal parts of ether and 95% ethyl alcohol. They were then stained with haematoxylin and eosin and mounted under a coverslip in DPX.

They were examined under low power, high power and oil immersion for cells resembling those of cytomegalic inclusion disease. If these cells were detected or there was reason to suspect, from the appearance and nature of the cells, that the patient might have cytomegalic inclusion disease, the other specimen, or preferably and usually a freshly collected specimen, was inoculated onto appropriate tissue-culture tubes.

#### *Tissue-culture Methods*

The studies of Rowe *et al.*, Margaret Smith, and Weller *et al.* had shown that this virus produced little or no cytopathic effect in tissue cultures of human epithelial cells, but did so in cultures of human fibroblasts. The strains they studied also produced no effect in cultures of various monkey tissues or in cultures of tissues from other animals. It was decided, therefore, to attempt the culture of the virus on cultures of uterine-muscle tissue and on cultures of fibroblast-like cells established in the course of other work. Arrangements were made with the Department of Obstetrics and Gynaecology of the University of the Witwatersrand and also with Dr. N. de la Hunt for a supply of tissue from patients subjected to hysterectomy.

The uterus was sent immediately after operation to the laboratory, where suitable segments of tissue were removed. These were minced finely with scissors and were then

trypsinized in 0·5% trypsin prepared as described by Dulbecco, for 3 hours, during which the tissue was agitated with a magnetic stirrer. The tissue was then spun for 5 minutes at 1,500 r.p.m., the supernatant decanted and the cells washed 3 times in Connaught medium 199. The cells were then suspended in a medium consisting of Connaught 199 and 20% human serum, and tissue-culture tubes were prepared. For the maintenance phase Connaught medium 199 and 5% bovine serum were used.

Two cell lines derived from eye tissue and established by Mrs. E. Cuthbertson, were also used in this study.

The first was derived from a human embryo eye received from Dr. H. H. Malherbe on 12 June 1959. This was minced and planted in clot culture prepared with fowl plasma and chick-embryo extract. On 18 June a good outgrowth of cells was apparent. The culture was then trypsinized and planted onto fresh tubes. Since then, weekly trypsinizations have been done and subcultures prepared from the resulting suspensions of cells. For the planting and outgrowth phase, Connaught medium 199 and 20% human serum, and for the maintenance phase, Connaught medium 199 and 5% bovine or horse serum were used. The cells of this line resemble fibroblasts in appearance.

The second line was derived from the tissue of a naevus of the eye removed at operation on 1 July 1959 by Dr. Graham Scott and sent that day in nutrient fluid to the laboratory. There it was minced with fine scissors and planted in clot culture with fowl plasma and chick-embryo extract. On 17 July a good growth had occurred and the culture was trypsinized and subcultures prepared. These also grew well, and since then weekly passages have been made. The medium used for the planting and growth phase was Connaught 199 and 20% human serum, and for the maintenance phase Connaught 199 medium and 5% bovine or horse serum. The cells of this line also resembled fibroblasts.

When a good confluent growth of tissue had been obtained, usually one week after planting, the nutrient medium was withdrawn and replaced by maintenance medium and the tubes were ready for inoculation. Each specimen of urine was inoculated into 2 or 3 tissue-culture tubes of each of the tissues. These tubes were examined daily. When the pH of the medium indicated the need, the medium was withdrawn and replaced by fresh. The tubes were incubated for periods of up to 30 days and sometimes longer. Coverslip cultures were also prepared for study of the detailed cytopathic changes.

*Results*

A large number of patients have been examined in the 2 years since this study was begun. From 2 of them a virus, resembling in its cytopathogenic properties the virus of cytomegalic inclusion disease, was isolated from the urine during life. One of these infants died and a post-mortem study confirmed the diagnosis. A detailed description of these cases, compiled by I.K. and R.S. follows:

*Case 1:*

J.G., a male infant, was born prematurely, birth weight 4 lb. 2¾ oz., after a normal pregnancy and labour. This was the mother's first pregnancy. The infant became jaundiced 3 days after delivery and this became noticeably worse on the following day when the total serum-bilirubin level rose to 21·9 mg. per 100 ml. with a direct bilirubin level of 2·5 mg. per 100 ml. The haemoglobin level was 22·5 G. per 100 ml. At this stage, since kernicterus was feared, an exsanguination transfusion was carried out, 520 ml. of blood being withdrawn and 515 ml. given. At the end, the total serum-bilirubin level was 6·6 mg. per 100 ml. with a direct level of 0·9 mg. per 100 ml. The following day the infant again developed an increasing jaundice and a second exchange transfusion was performed, when the total serum-bilirubin level was 19·8 mg. per 100 ml. Subsequent to this transfusion the baby improved and lost all evidence of clinical jaundice. The baby was Group O, Rh positive, and the Coombs' test gave a negative result. The mother's blood was Group O, Rh positive and had no abnormal antibodies. The Wassermann reactions of mother and child were negative.

At the end of the second week after birth the infant again developed icterus, gradually increasing in intensity and presenting a picture of an obstructive jaundice with dark urine and pale stools. No reducing substances were found in the urine on repeated examination, thus excluding galactosaemia as a cause of the condition. Bilirubin was consistently present.

The blood count was essentially normal, with normal platelets. The osmotic fragility of the red cells was within the normal range. A random blood-sugar estimation was normal.

The serum proteins showed a total of 5·3 G. per 100 ml. with the following electrophoretic pattern (all figures in grams per 100 ml.): albumin 2·67, alpha$_1$ globulin 0·62, alpha$_2$ globulin 0·65, beta$_0$ globulin 0·58, and gamma globulin 0.78.

The liver-function tests gave the following results: thymol turbidity 1 unit, thymol flocculation negative, colloidal-red flocculation negative, cephalin-cholesterol flocculation negative, zinc-sulphate turbidity 4·2 units, alkaline phosphatase 33 units, cholinesterase 61% of average, and serum G - O transaminase 320 units per 100 mg.

An intravenous cholecystogram was performed, but the biliary system was not seen, suggesting either poor function or an atresic biliary-duct system. The total serum-bilirubin level showed a steady increase to an ultimate level of 17 mg. per 100 ml. (direct 8·5 mg. per 100 ml.). At this stage an exploratory laparotomy was contemplated in the hope of relieving a congenital obstruction to the extrahepatic biliary ducts. Fortunately, before this was done the jaundice began to subside.

The diagnosis of cytomegalic inclusion disease was suggested at this stage, based on the result of the microscopic examination of the urine. This showed many cells with clearly defined, round, eosinophil inclusion bodies in the cytoplasm, and some cells with ballooning of the nucleus, margination of the chromatin and intranuclear eosinophil inclusion bodies.

A virus resembling the virus of cytomegalic inclusion disease was isolated in tissue culture from a freshly collected specimen of urine.

A course of prednisone was started and continued for 6 weeks. The icterus gradually subsided, the stools became normal in colour, and the urine free from bilirubin. The patient continued to make good progress, but when 9 months old still had a slight hepatosplenomegaly.

*Case 2*

D.S.K., a male infant of European descent, was born at home after a full-term normal pregnancy and delivery, with a birth weight of 7¼ lb. He was the 10th child of the mother, whose other infants had not been jaundiced. He was admitted to hospital 5 days after birth because of increasing jaundice, which had first been noticed on the 3rd day. His spleen was palpable. The total serum-bilirubin level was 27·6 mg. per 100 ml. with a direct-bilirubin level of 1·4 mg. per 100 ml. Because Rh incompatibility was suspected and kernicterus was feared, an exsanguination transfusion was performed; 760 ml. of blood was withdrawn and 750 ml. given. After this transfusion the total serum-bilirubin was 8·9 mg. per 100 ml. and the direct-bilirubin level 0·6 mg. per 100 ml. The level of total serum bilirubin rose rapidly again, and 15 hours later, when the total bilirubin level had reached 23·2 mg. per 100 ml. with a direct level of 1·1 mg. per 100 ml., a second exchange transfusion was done.

Laboratory studies showed no evidence of a haemolytic process, the mother was found to be Group A, Rh positive, and had no abnormal antibodies. The patient was Group O, Rh positive, and the Coombs' test gave a negative reading. The Wassermann test gave a negative result. The total serum-protein level was 4.8 per 100 ml., albumin 2·3 G. per 100 ml. and globulin 2·5 G. per 100 ml.

This infant subsequently failed to thrive. He had periods of vomiting and often had loose stools. He was given a course of prednisone therapy, but without benefit. His weight gradually decreased until it fell to 4½ lb. He died when 3 months old.

The diagnosis of cytomegalic inclusion disease was suggested shortly before his death by the study of smears made from the urine. These showed many epithelial cells, some of which contained clearly defined, round, eosinophil inclusions in the cytoplasm, a few showed margination of the chromatin of the

nucleus and intranuclear eosinophil inclusion bodies. These findings were considered to be in keeping with the diagnosis of cytomegalic inclusion disease.

A virus resembling the virus of cytomegalic inclusion disease was isolated from the specimen of urine collected at the same time.

An autopsy was performed, and showed a grossly wasted infant with little subcutaneous fat and generally congested organs. Histological examination of sections from the viscera revealed typical cells of cytomegalic inclusion disease in the tubular epithelium of the kidney, the cells lining the alveolar spaces of the lung, the acini and the ducts of the pancreas, and the epithelium lining the thyroid follicles. Cytomegalic cells were not found in the liver, spleen, stomach, heart, thymus or brain. Salivary-gland tissue was not taken for study. Other findings of note were atrophy of the lymphoid tissue of the spleen and thymus.

*Comment*

These 2 cases illustrate some of the manifestations of cytomegalic inclusion disease occurring in the neonatal period. Both presented with jaundice, at first naturally suspected of being caused by Rh incompatibility. Both, in view of the deepening jaundice, were given exchange transfusions because it was feared they might develop kernicterus. Later, because of the failure of the jaundice to clear, congenital atresia was suspected in one case. This patient has recovered and, but for the persistence of hepatosplenomegaly, he is apparently in good health and is developing normally. The other infant died when 3 months old after a progressive deterioration.

In both cases the diagnosis was established during life by the examination of the urine. Microscopic study revealed a cytological picture suggestive of cytomegalic inclusion disease, and this suspicion was confirmed by the isolation of a virus in tissue culture.

*Virus Studies*

Tissue cultures of the human embryo eye cell line and of the human conjunctiva cell line were inoculated with samples of the respective specimens of urine. Islands of rounded, slightly refractile cells appeared in the sheet and gradually increased in size by peripheral extension. Several islands appeared in each tube and then gradually extended, and finally involved the whole sheet of cells.

The coverslip cultures prepared were stained with haematoxylin and eosin. These showed the characteristic appearance of cytomegalic inclusion disease and all stages in the evolution of the lesion of the cell could be found. The islands of altered cells were clearly differentiated from the surrounding sheet of apparently normal cells. The earliest change appeared to be the rounding of the cell associated with an eosinophilic change in the cytoplasm. The area of eosinophilia increased, and an ill-defined mass formed and finally filled most of the cytoplasm, indenting and often displacing the nucleus to one end of the cell. In many, but not in all, affected cells the characteristic intranuclear body developed. This first appeared as a small eosinophil dot in the middle of the nucleus, but distinct from the nucleolus which sometimes showed enlargement and distortion. The inclusion increased in size until it almost filled the nucleus, but was separated from the marginated nuclear membrane by a clear halo. It assumed the outline of the nucleus, was often rounded, but was sometimes elongated and kidney-shaped, and occasionally formed 2 rounded bodies separated by the nucleolus, giving a spectacle appearance. In some cells a number of small separate eosinophil bodies, each surrounded by a halo, occurred in the nucleus. Occasionally a large cell with 2 or more nuclei, each containing characteristic intranuclear inclusions with a rounded eosinophilic mass in the cytoplasm, was seen. The intranuclear inclusion body often showed internal structure. In some this suggested a number of relatively large subunits, each consisting of a central dot surrounded by a clear circular zone. In others the appearance was granular and suggestive of a mass of elementary bodies.

Attempts were made to grow the virus on a number of other tissues, including human amnion, primary monkey (*Cercopithecus aethiops pygerythrus*) kidney, and guinea-pig heart-cell line tissue cultures. These cultures revealed no cytopathic effects.

In their cytopathic effects and the appearance of the intranuclear inclusion bodies in tissue cultures of human fibroblasts, and in their host range of tissue-culture cells, the viruses isolated from these 2 patients resembled the Davis strains of virus received from Professor Weller (Figs. 1 - 6). Studies of their immunological relationships are in progress.

There is some resemblance between the lesions produced by these viruses and those produced by herpes simplex virus. However, the latter are usually much more rapid in their evolution and in the destruction of the tissue culture. The herpes virus also has a much wider range of host tissue-culture cells, being cytopathogenic to human epithelial tissues, monkey, rabbit and chick tissues and a number of others, as well as to human fibroblast tissue cultures.

It was concluded, therefore, that the viruses isolated were strains of the cytomegalic inclusion disease virus.

## DISCUSSION

The high proportion of cases of cytomegalic inclusion disease ascribed in the earliest writings to congenital syphilis raises doubts about the correctness of this suspicion. Indeed, in the light of subsequent developments and of present knowledge, it seems certain that in most instances this diagnosis was wrong and that possibly unnecessary mental anguish was caused to the parents by these errors. On the other hand, at the present time cases of haemolytic anaemia of the newborn and of *icterus neonatorum* are usually suspected of having a background of Rh incompatibility between the mother and baby. In many instances, of course, this interpretation is justified and can be confirmed by suitable laboratory tests. In others, no incompatibility is found and it is in such cases in particular that generalized cytomegalic inclusion disease should be considered and excluded. The distinction is important because the treatment and prognosis are different.

It is, therefore, of interest to note that the 2 patients from whom virus was isolated in the present study were both in the first instance suspected of having haemolytic disease of the newborn, presumed to be caused by Rh incompatibility between the mother and the baby, and both babies received exchange transfusions.

In the first patient, an infant born prematurely with a birth weight of 4 lb. 2¼ oz., presenting with increasing jaundice, it was shown that both mother and child were Group O, Rh positive and that the mother had no abnormal antibodies. The possibility of atresia of the biliary ducts was then considered, but at 2 months of age the jaundice began to subside. Also, at this time the examination of the urine microscopically revealed the presence of cells showing ballooning of the nuclei with margination of the chromatin and eosinophil intranuclear inclusions suggesting cytomegalic inclusion disease. This suspicion was confirmed by the isolation of the virus from the urine.

At 9 months of age this infant still had an enlarged liver and spleen, but was now thriving. These infections, presumably occurring *in utero* before birth, are thus not necessarily fatal or associated with grave deformities, and indeed may have a favourable outcome.

The second patient, a male infant, born at full term with a birth weight of 7¼ lb., also presented with increasing jaundice which had become apparent 3 days after birth. Rh incompatibility was again suspected and an exchange transfusion was given. However, the baby was

found to be Group O, Rh positive and to give negative reactions in the Coombs' and Wassermann tests. The mother was found to be Group A, Rh positive and to have no abnormal antibodies. The baby failed to thrive.

Examination of the urine when he was 3 months old revealed a picture suggestive of cytomegalic inclusion disease.

The baby died soon afterwards and the histological

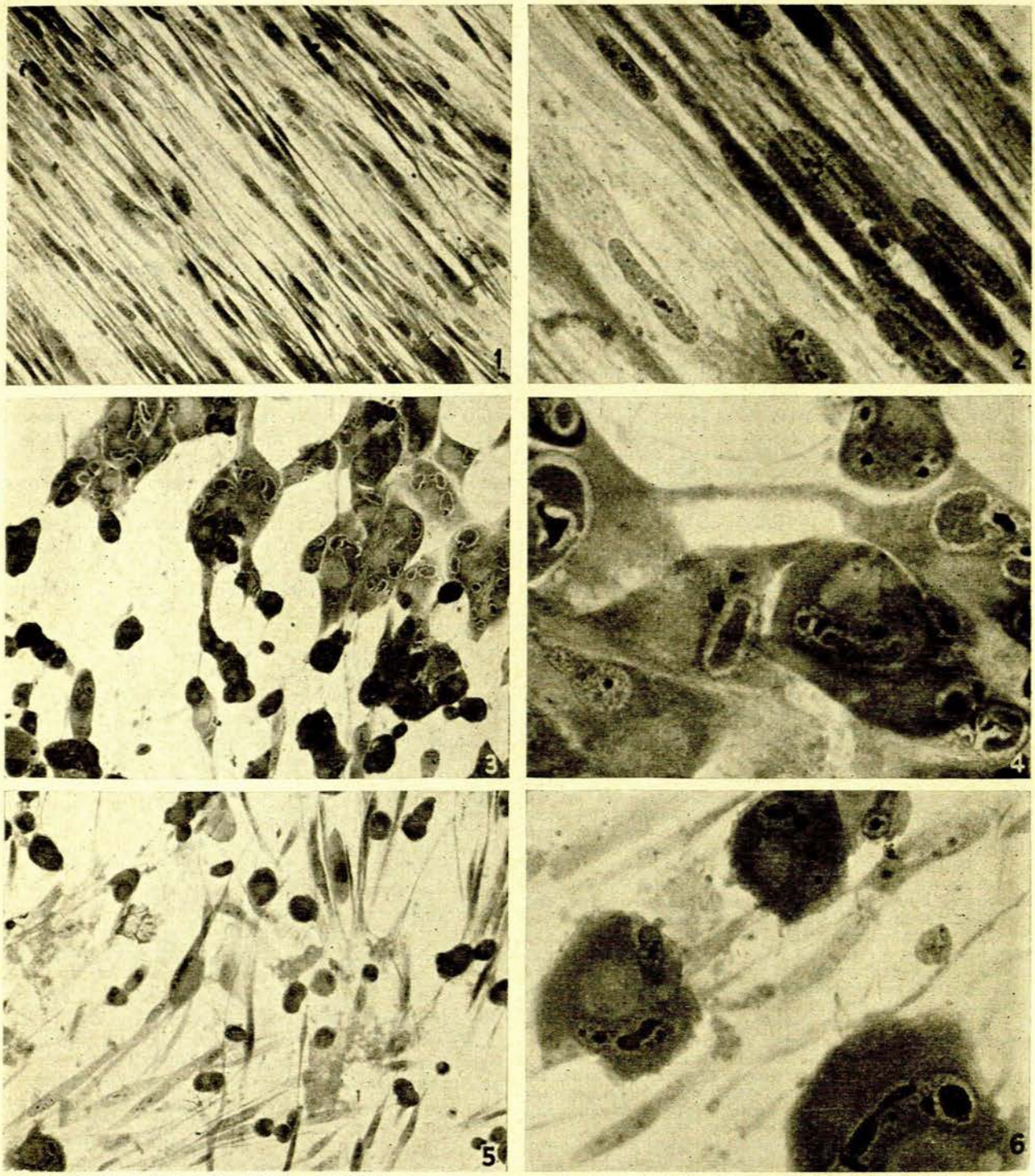

*Fig. 1.* Culture of human fibroblasts (normal × 120).
*Fig. 2.* Culture of human fibroblasts (normal × 480).
*Fig. 3.* Culture of human fibroblasts infected with Weller's 'Davis' strain (normal × 120). Note rounded cells and intranuclear inclusion bodies.
*Fig. 4.* Culture of human fibroblasts infected with Weller's 'Davis' strain (normal × 480). Note characteristic inclusion bodies.
*Fig. 5.* Culture of human fibroblasts infected with South African strain (normal × 120). Note rounded cells and intranuclear inclusion bodies.
*Fig. 6.* Culture of human fibroblasts infected with South African strain (normal × 480). Note characteristic intranuclear inclusion bodies.

sections of the organs removed postmortem showed typical cytomegalic inclusion cells in epithelial cells of several organs, thus confirming the diagnosis.

Tissue cultures, inoculated with a suspension prepared from the spleen, developed typical lesions of cytomegalic inclusion virus infection.

Both these cases exhibit the value of the examination of the urine in giving a lead to the diagnosis, and of the value of tissue-culture isolation and identification of the virus in establishing the cause of the patients' illness.

No serological studies were carried out in the present investigation, except to note that the convalescent sera from baby G and his mother neutralized the virus in a tissue-culture neutralization test. Since no pre-illness phase sera were available for testing, the time of development of these antibodies is unknown. Such information in the case of the mothers would indicate whether infection was acquired during pregnancy or whether the infection was of longer standing.

It is not yet clear whether only one pregnancy or whether the babies of successive pregnancies may be affected. Most recent studies suggest that only one pregnancy is involved and that subsequent infants are not affected. However, in some of the earlier reports more than one sibling had been affected by a condition possibly caused by cytomegalic inclusion disease. This question is therefore in need of further investigation and, of course, should soon be answered by continued observation of the subsequent siblings of affected babies.

It is known that the excretion of the virus in the urine of affected children may continue for many months, and presumably could be a source of infection to other infants, children and even adults in the same household or institution.

Other studies have revealed that the proportion of individuals with protective antibodies to the virus increases with increasing age, and that about 50% of adults in some cities of North America have acquired immunity. This infection is thus very prevalent, a conclusion already drawn from the results of postmortem studies of infants dying from a variety of causes. Fortunately it is also clear that, of those who acquire the infection, only a few suffer serious ill-effects, but in some of these the disease may progress and end fatally.

It is particularly dangerous when the infection is acquired *in utero* or in the neonatal period. As a cause of congenital defects and of serious neonatal disorders, cytomegalic inclusion disease rivals toxoplasmosis and Rh incompatibility in importance. Every effort should therefore be made to determine its epidemiology and to find tests for the earlier recognition of infection in potential and pregnant mothers, and to find the means of treatment and prevention of the infection.

SUMMARY

The literature concerned with cytomegalic inclusion disease or salivary-gland disease is briefly reviewed. Evidence of the prevalence of this infection is given by the finding of the typical inclusions in the salivary glands of 10 - 32% of infants examined postmortem regardless of the cause of death. The infection rarely causes overt disease except in the neonatal period and in infants below 2 years of age. However, in this age group it may cause a progressive illness which often ends fatally. It is also an important cause of congenital defects ranking with toxoplasmosis in this respect.

The association of this infection with haemolytic disease and jaundice of the newborn has been noted by a number of investigators. It was suggested that the widespread lesions found in some infants were from the dissemination of a human strain of the salivary-gland virus.

The affected cells are epithelial cells, and are usually considerably enlarged. They contain eosinophil intranuclear inclusion bodies separated from the marginated chromatin by a clear halo, giving a typical bird's eye appearance. Cells characteristic of the disease may be found in the urine, making it possible to confirm a clinical diagnosis of this condition by the microscopical examination of smears.

Cytomegalic inclusion disease affects various species of animals, but the strains of virus appear to be host-species-specific. Several have now been successfully cultured in tissue cultures. Human strains have been grown, and their cytopathogenicity studied in tissue cultures of human fibroblasts.

Two patients whose illness was shown to be caused by cytomegalic inclusion disease were admitted to the Transvaal Memorial Hospital for Children soon after birth. Both presented with increasing jaundice and it was thought that their condition was the result of Rh incompatibility. Both were given exchange transfusions. One has recovered and is now developing normally. The other died after a progressive loss of weight and deterioration in his condition.

The diagnosis was suggested by the study of urinary smears, and in each case was confirmed by the culture of the virus in tissue cultures of human fibroblasts, and in the fatal case by postmortem studies.

The importance of this condition is discussed and the need for early recognition of the infection and for means of preventing its serious manifestations is emphasized.

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