Ryan Virus: a Possible New Human Pathogen

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In November 1963 a cytopathic agent was isolated from a baby who died in the Royal Manchester Children's Hospital. This agent produced an unfamiliar effect in cell cultures, and further studies confirmed that this effect was unlike anything previously encountered by us. Since then five other agents, producing similar cytopathic effects in cell cultures, have been isolated in the Public Health Laboratories at Manchester, Coventry, Cirencester, and Colindale. This paper describes the properties, so far as they have been determined, of these agents and the clinical features of the illnesses in the patients from whom they were isolated. We shall refer to them as Ryan viruses, after the name of the child from whom the first strain was isolated, and because the properties so far determined suggest they are viruses rather than some other micro-organism.

Details of Virus Isolation

Ryan 1

This strain was isolated from a post-mortem bronchial swab of a male infant aged 7 months who, six days before admission to hospital, developed a sore throat and ulcers in the mouth which later spread to the face. He was obviously unwell, was unable to suck, and his stools were loose. On the day before admission he began to vomit and at the same time developed a cough. He became drowsy and dyspnoeic, and when admitted to hospital on 31 October 1963 was moribund. He had Cheyne-Stokes respiration, scanty coarse rales in his chest, and made intermittent jerky movements.

Lumbar puncture produced a clear cerebrospinal fluid with no pleocytosis. The blood count was normal. X-ray examination of the chest revealed some patchy consolidation with dilatation of the right side of the heart. Next day the temperature, which had been normal on admission, rose to 102° F. (38.9° C.). The child became apnoeic and deeply cyanosed, and died in spite of stimulants, fluid therapy, hydrocortisone, penicillin, and tetracycline.

At necropsy there were no gross macroscopic changes apart from some emphysema, petechiae and small areas of congestion in the lungs, a fatty liver, prominent mesenteric nodes, and mucopus in the middle ears. Bacteriological cultures yielded *Escherichia coli* from the ears.

Microscopical examination of the lungs showed small scattered areas of congestion and alveolar haemorrhage. There was a follicular reaction in the spleen and lymph nodes. The liver showed swelling and vacuolation of parenchymal cells at the periphery of the lobules, with widespread damage to nuclei but no definite inclusion bodies. Small focal haemorrhagic areas of mixed-cell infiltration with lymphocytes predominating were noted. There were several small cellular foci of lymphocytes and glial cells in the medulla but not elsewhere in the brain. No unequivocal inclusions were seen. The facial lesions were not examined virologically. Death appeared to be due to a respiratory infection associated with encephalitis and hepatitis.

The virus was isolated in HeLa-cell cultures grown and maintained as already described (Manchester Report, 1964).

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Ryan 2

This strain was isolated in February 1964 from a man aged 29 who was taken ill with an acute influenza-like illness while at work in an aircraft factory. The agent was isolated in HeLa cells, handled by a method similar but not identical to that already mentioned for Ryan 1 (M.R.C. Report, 1965).

Ryan 3

This strain was isolated in March 1964 from a woman aged 30 with an acute influenza-like illness which started with generalized aching. Severe coryza and a mild cough developed during the next two days. She probably had a slight fever, but by the third day her temperature had subsided. A small herpetic lesion was noted in her right nostril. Her husband was in bed at the same time with a similar disease.

The strain was isolated in rhesus-monkey-kidney cultures grown and maintained as described in the M.R.C. Report (1965).

Ryan 4

This strain was isolated from a girl aged 10 years who was ill with fever, cough, mild generalized lymphadenopathy and splenomegaly, and some vomiting for four or five days in April 1964. A few days later her mother suffered from a similar illness.

The virus was isolated in rhesus-monkey-kidney-cell cultures grown and maintained as described previously (Manchester Report, 1964).

Ryan 5

This strain was isolated from a 2-year-old girl who was admitted to hospital in September 1964 suffering from fever and convulsions. Except for some congestion of the throat, no other symptoms were noted besides the convulsions.

The virus was isolated in rhesus-monkey-kidney-cell cultures (see Ryan 4).

Ryan 6

This strain was isolated from a child aged 4 years who became ill in March 1965 with low fever and cough. Rales and rhonchi were heard in the chest, and she was admitted to hospital with a diagnosis of bronchitis. Recovery was fairly rapid and she was discharged within the week. Her sister, aged 2 years, was ill at the same time with a similar clinical condition. From both these children respiratory syncytial virus was isolated in monkey-kidney cultures.

The virus was isolated in HeLa cells (see Ryan 2).

Properties of Ryan Virus

Host Range

The strains of Ryan virus have been isolated in both HeLa and rhesus-monkey-kidney-cell cultures. They also produced

a cytopathic effect in cultures of human-embryo kidney, Hep 2, KB, ARK.13 strain of rabbit-kidney cells, human thyroid and amnion cells, and in human diploid embryo-lung-cell strains. On primary isolation the cytopathic effects developed in from four days to the third week of incubation, but subsequently appeared within three to five days after inoculation of undiluted cell-culture suspensions. These effects consisted initially of focal lesions at the lower end of the cell-culture tube, which then spread throughout the culture. Sometimes they started on the edges of the cell sheet with rounding up and sharpening of the cell outline, an appearance not unlike that caused by adenoviruses. The most characteristic effect was the appearance of discrete shrivelled crenated cells adhering in large numbers to the glass after the cytopathic process had become complete (Fig. 1). These cells were often bent in shape and had the appearance of miniature loafahs. With moderate to high doses of virus complete destruction of the cell sheet occurred within the next 24 to 36 hours; but with one strain, Ryan 5, the cytopathic effect was not always complete when low doses were used for inoculation of cell cultures.

The virus could be transmitted serially with ease provided cellular material remained in the fluid. Titres were moderately high, 10^5 – 10^6 /ml. being quite usual. The cytopathic effect was more pronounced in continuous cell lines than in primary cultures and was similar in each of the lines tested. Growth in monkey-kidney cells was a little more erratic and varied from culture to culture. Human amnion cells were very variable in their ability to support growth of these viruses, but all strains

FIG. 1.—HeLa cells. Four days after infection with Ryan virus. ($\times 85$.)

FIG. 2.—HeLa cells 48 hours after infection with Ryan virus. Stained with haematoxylin and eosin. ($\times 560$.)

grew well in human thyroid cultures. Coverslip preparations of cells fixed after 24, 48, or 72 hours' incubation showed a variety of changes in the cell nuclei, though cultures appeared quite normal when unstained. One noticeable change was the presence of small round eosinophilic inclusion bodies, either single or multiple, surrounded by clear haloes (Fig. 2). These bodies were quite distinct from the nucleoli. In other cells the nuclei had a generally disorganized structure, and contained many round clumps of fragmented chromatin which were often margined. Some cells showed mixtures of inclusion bodies and fragmented chromatin, while others remained normal. Herpes-like inclusions were not seen.

The inoculation of four strains of Ryan virus into both suckling and adult mice by the intraperitoneal as well as the intracerebral route caused signs of encephalitis. Suckling mice became ataxic and excitable within three to five days with tremors and convulsive movements, and usually died two or three days after the onset of illness. In some adult mice the symptoms were more prolonged and not always fatal. This effect could be irregularly transmitted to further litters by the passage of homogenates of brain and torso. The virus could also be recovered in tissue culture from such homogenates.

Three strains of Ryan virus were tested on the chorioallantoic membrane of fertile eggs, and Ryan 1 in the amniotic and allantoic cavities of the developing chick embryo. No specific lesions were found. No lesions were produced in rabbits or guinea-pigs by subcutaneous or intravenous inoculations. Intracerebral inoculation of two cynomolgus monkeys produced no obvious illness.

Other Properties of Ryan Virus

Filtration experiments to determine size were unsuccessful, as the virus could not be separated from the debris of any of the cultures in which they multiplied. All were deposited by one or two centrifugations at 3,000 to 3,400 r.p.m. for 15 to 30 minutes, the supernatant being free of infectivity. The resuspension of the cell deposit to the original volume produced cytopathic effects to the same titre as the original. Five attempts to pass these agents through gradocol membranes were unsuccessful except in one instance. Here a suspension of Ryan 1 with a titre of 10^6 /ml. before filtration produced a low titre ($10^{1.5}$ /ml.) after passing through a gradocol membrane with a minimum pore diameter of 330μ . The same membrane held back vaccinia virus completely. This would suggest that Ryan 1 was less than 150 – 200μ in diameter.

Treatment of these viruses with 20% ethyl ether for 18 hours at 4°C . completely abolished their infectivity.

Ryan 1 and 5 were tested for resistance to acid and found to withstand pH 4 for four hours without loss of titre.

Ryan 1 was inactivated at 56°C . in a water-bath within half an hour, but at 37°C ., after an initial hundredfold drop in titre during the first 72 to 96 hours, the infectivity decreased about tenfold during the next week. Repeated cycles of freezing and thawing resulted in a tenfold to hundredfold drop in titre.

Ryan 1 appeared to be inhibited by 5-fluoro-deoxyuridine at a concentration of 0.01 mg./ml., this effect being counteracted by thymidine used at the same concentration. However, neither Ryan 1 nor Ryan 5 was inhibited by 5-iodo-deoxyuridine with concentrations as high as 0.2 mg./ml., and irrespective of the dose used. Neither was there any reduction in titre in the cell cultures treated with 5-iodo-deoxyuridine. In these tests strains of herpes simplex virus, adenovirus, vaccinia virus, and poliovirus were tested concurrently and behaved as expected, the growth of all but poliovirus being inhibited by these compounds, with thymidine reversing this inhibition.

Cell cultures infected with these viruses failed to haemadsorb with sheep, fowl, human group O, rat, monkey, or guinea-pig red cells. Neither was haemagglutination by suspensions of virus observed with these red cells.

Electron micrographs of cell cultures and of deposits failed to demonstrate any definite viral bodies, although intranuclear inclusions were recognizable.

Serological tests gave very erratic results and were difficult to interpret because of the close association with cells. No neutralization of Ryan virus was found with antisera to herpes simplex, E.C.H.O. virus 1-30, poliovirus, or Cocksackie virus; nor was it neutralized or inhibited by normal calf, horse, or human sera. Attempts to prepare a complement-fixing antigen have been unsuccessful so far.

Paired sera were available from three of the patients, but again equivocal results were obtained in neutralization tests with Ryan virus. However, none of them had developed complement-fixing or neutralizing antibody to herpes simplex.

Attempts to Separate Virus from Cell Debris

The serial transfer of Ryan virus was not dependent on the viability of the cells, as infection could be transmitted with cell debris. Efforts were made to separate infective particles from this cell debris by various means. Debris from infected HeLa-cell cultures was submitted to ultrasonic disintegration for periods of 15 seconds to three minutes; by up to four cycles of freezing and thawing; by the use of a Tenbroeck grinder and an M.S.E. homogenizer. Cell-free infective material was not obtained by any of these methods, nor could it be demonstrated after treatment with other reagents such as 1% crystalline trypsin for one and a half hours, 0.25% Difco trypsin (1/250) for one to three hours, by phage lysis, streptomycin enzyme, 1% pronase, and 4M urea.

Because it was hoped that Ryan virus would behave in thyroid-cell cultures like varicella-zoster virus, the method of separation described by Caunt and Taylor-Robinson (1964) for the latter virus was tried with Ryan 1 and 5. However, no infective material was found in the supernatant fluids of large pools of infected thyroid-cell suspension either before or after ultrasonic treatment or repeated freezing and thawing.

Tests for Presence of Mycoplasma or Bacteria

First monkey-kidney-pass material of Ryan 3, which had been isolated in monkey-kidney cultures, was tested for evidence of mycoplasma on PPLO-agar incubated in air for three weeks and in increased CO₂ for 10 days with negative results. Kanamycin or neomycin 100 mg./ml. in the medium did not affect the growth of these strains even if these drugs were added to a virus suspension and left for one and a half hours before inoculation of cell cultures. Broth and blood-agar plates inoculated with cell-culture material failed to reveal the presence of any bacteria.

Discussion

The six agents now reported have enough properties in common to be included in a single virus group. Their cytopathic effect and inclusion bodies were similar but differed from those described for other cytopathic agents. All strains were associated intimately with cellular material, being deposited with the cell debris even with low-speed centrifugation, and all were ether-sensitive.

The different results obtained with 5-fluoro- and 5-iodo-deoxyuridine may be explained by the fact that these substances may act differently on the cycle of nucleic acid formation (Salzman *et al.*, 1963; Easterbrook and Davern, 1963; Stevens, 1965). The inhibition by the fluorine compound and the reversal by thymidine would indicate that these viruses contained deoxyribonucleic acid (D.N.A.) or that some step in their multiplication is D.N.A.-dependent. The effect on

suckling and adult mice was not unlike that produced by herpes simplex virus.

These properties could be interpreted most reasonably at present as those of a virus and one which was ether-sensitive and contained deoxyribonucleic acid. Its behaviour indicated that it might belong with the herpes viruses lying intermediate between herpes simplex and varicella-zoster-cytomegalovirus, since it has the ability to grow in a wide range of tissue cultures and is pathogenic for mice like herpes simplex but is avidly cell-bound and fails to produce pocks on the chorioallantoic membrane like varicella-zoster-cytomegalovirus (see Table).

Relation of Ryan Virus to Herpes Virus Group

	Herpes Simplex	Ryan	Varicella-Zoster Cytomegalovirus
D.N.A.	+	? +	+
Ether-sensitivity . . .	+	+	+
Growth on chorioallantoic membrane	+	—	—
Association with cells	Readily released	Avidly cell bound	Cell bound but may be released
Inclusions	Basophilic or eosinophilic 1 or 2	Eosinophilic intranuclear multiple	Eosinophilic intranuclear 1 or 2
Mouse pathogenicity	+	+	—
Growth in tissue culture	Wide range in primary cultures and cell lines	Wide range in primary cultures and cell lines	Restricted to primary cultures

However, against this is the failure to find any viral bodies characteristic of this group in many careful searches of thin sections of infected cells. As our knowledge of the properties of this agent is incomplete there is always the possibility that it is an antibiotic-resistant micro-organism, other than a virus, which cannot be detected by the cultural methods employed.

It is hoped that the description of this agent with its unusual and characteristic cytopathic effect in tissue cultures will allow its recognition in other laboratories, as so far only a few strains have been isolated and their association with any particular disease is uncertain.

Summary

Six strains of an agent which produced unusual cytopathic effects in a variety of cell cultures were isolated in four different laboratories from patients with respiratory or more generalized infections. The clinical features of their illnesses and the general properties of the agents are described.

Serological investigations have been hindered by the difficulty encountered in separating the agent from the cellular debris of the cultures.

The properties of this agent as determined so far are compatible with it being a virus, perhaps related to the herpes virus group, but its exact identity has not yet been defined.

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REFERENCES

- Caunt, A. E., and Taylor-Robinson, D. (1964). *J. Hyg. (Lond.)*, **62**, 413.
 Easterbrook, K. B., and Davern, C. I. (1963). *Virology*, **19**, 509.
 Manchester Report (1964). *Mth. Bull. Minist. Hlth Lab. Serv.*, **23**, 136.
 M.R.C. Report (1965). *Brit. med. J.*, **2**, 319.
 Salzman, N. P., Shatkin, A. J., and Sebring, E. D. (1963). *Virology*, **19**, 542.
 Stevens, J. G. (1965). *Ibid.*, **25**, 379.