The incidence of infection with cytomegalovirus in a normal population

A serological study in Greater London

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Disease caused by cytomegaloviruses occurs in its most severe and characteristic form in the neonatal period. The majority of these cases acquire their infections *in utero* and the clinical features, which are present at birth or appear shortly afterwards, include jaundice with hepatosplenomegaly, thrombocytopenic purpura, erythroblastic anaemia, pneumonitis and often evidence of neural damage which may be associated with microcephaly and cerebral calcification. This condition was previously believed to be almost always fatal, but it is now known that recovery does occur although it may be followed by residual abnormalities such as mental deficiency, microcephaly, hydrocephalus, epilepsy, cerebral palsies and optic atrophy (Birdsong, Smith, Mitchell & Corey, 1956; McElfresh & Arey, 1957; Weller & Hanshaw, 1962; Medearis, 1964). When older infants are infected the clinical pattern is ill-defined and usually takes the form of an unresolving interstitial pneumonia or sometimes intractable gastro-intestinal symptoms, often with evidence of hepatic or renal dysfunction (Smith & Vellios, 1950; Wyatt, Sexton, Lee & Pinkerton, 1950; Medearis, 1957). These post-natal infections tend to be superimposed on underlying serious debilitating diseases. The true incidence of the neonatal and post-natal diseases is uncertain, since many of the fatal cases and of those that recover are undiagnosed. Fatal cases, however, have accounted for 1–2 % of unselected paediatric autopsies in Boston and St Louis in the United States, as well as in Finland and Germany (Farber & Wolbach, 1932; McCordock & Smith, 1934; Wyatt *et al.* 1950; Ahvenainen, 1952; Seifert & Oehme, 1957). In other parts of the United States and also in Great Britain they seem to be very much less common (Potter, 1957; Symmers, 1960; Crome & France, 1959).

After 4 years of age the disease is very rare. Most cases, in children and adults, have been recognized unexpectedly at post-mortem or in biopsy specimens taken for other purposes (Wong & Warner, 1962). It occurs mostly as a complication of underlying chronic debilitating conditions such as leukaemia and lymphoma which depress the normal defence mechanisms of the body, especially when steroids and cytotoxic drugs have been used in treatment.

Although clinical disease is infrequent it has been known for some time that a more common but inapparent or symptomless form of infection occurs in early childhood. This is revealed by the presence of typical cytomegalic cells in the

salivary glands and occasionally also in the kidneys. Such cells are an incidental finding at post-mortems carried out on children who have died from other causes, and are rarely seen under 2 months or after 2 years of age. They have been described in 10–12 % of unselected paediatric autopsies in Germany and the United States (Löwenstein, 1907; Seifert & Oehme, 1957; Farber & Wolbach, 1932; McCordock & Smith, 1934) and in as many as 18 and 32 % of autopsies in South America and Indonesia respectively (Potenza, 1954; Prawirohardjo, 1938). In Great Britain the incidence has been 5 % or less (Baar, 1955; McDonald, personal communication). Serological studies, which became possible after the isolation of cytomegaloviruses in tissue culture (Rowe et al. 1956; Smith, 1956; Weller, Macauley, Craig & Wirth, 1957), have confirmed that infection as opposed to disease is common in children. They have also shown infection to be common in adults, the incidence of antibodies increasing progressively with age to as high as 81 % after 35 years of age in the Washington area (Rowe et al. 1956).

The present paper is a serological study of infection in the London area, using the complement fixation method.

METHODS

Virus strains. These were 'Kerr', 'Davis' (Weller et al. 1957) and 'Ad 169' (Rowe et al. 1956) isolated in the United States, and 'Aravi', '138', 'Rawles' and 'Sh' isolated in this country (Stern, Lambert & Shakespeare, 1963). They were grown in human embryonic fibroblast tissue cultures. In the early part of the investigation these cultures were prepared as primary outgrowths in plasma clot; later semi-continuous diploid cell lines were used (Hayflick & Moorhead, 1961). The plasma clot cultures were incubated in roller tubes at 37° C. and both the growth and maintenance media were Earle's saline containing 0·5 % lactalbumin hydrolysate, 10 % inactivated horse serum, 0·01 % soy bean trypsin inhibitor and 200 units per ml. each of penicillin and streptomycin. The pH was adjusted with 5 % sodium bicarbonate to about 7·2. The diploid cultures were grown in stationary tubes in Eagle's medium with 10 % newborn calf serum and antibiotics and bicarbonate as above, and then maintained on medium 199 with 2 % newborn calf serum. The viruses were passaged by scraping infected cells off the glass into the medium, grinding the cell suspension for 15–20 sec. in a Ten Broek grinder and inoculating 0·1 ml. of ground suspension into fresh tissue culture tubes. Tissue culture-adapted strains, when kept in continuous passage without intervening storage, caused complete destruction of the cell sheet in 5–10 days. Less well-adapted strains required as long as 21–28 days or more.

Complement-fixation tests. Antigens were prepared by inoculating tubes containing fully grown sheets of fibroblasts with 0·1 ml. of ground infected cell suspension. The maintenance medium was changed after 2 days and subsequently usually at 3-day intervals. When the sheets showed almost complete cytopathic effects the cells were scraped into the medium. Pooled cell suspensions were centrifuged at 1500 r.p.m. for 10 min. and the deposited cells resuspended in veronal-buffered saline to one-fifth of the original volume. The cells were then disrupted by thorough grinding in chilled glass grinders or by exposure for 5 sec.

to ultrasonic vibrations. The treated suspension constituted the antigen and was kept frozen at $-70°$ C. in 1 ml. amounts in neutral glass ampoules. Just before use the antigen was heated at $56°$ C. for half an hour. Uninoculated tubes from the same batch of tissue culture were treated in the same way for use as controls. Each batch of antigen was examined in a chess-board titration against a standard positive pooled human serum. The antigen was used in the c.f. test at a dilution containing 2 units per 0·1 ml. Most preparations contained 2 units at a dilution of 1 in 2 or 1 in 4 and were free of anticomplementary activity at these dilutions. They maintained their potency at $-70°$ C. for at least 1 month. Latterly, the infected tissue culture cells were resuspended in veronal-buffered saline containing 25 % sorbitol before disruption. Such preparations retained unaltered c.f. activity for longer periods (Medearis, personal communication).

In the c.f. test all sera were inactivated at $56°$ C. for half an hour. Tests were carried out in Wasserman tubes, using 2 units of complement and overnight fixation at $4°$ C. Sera were first examined at a dilution of 1 in 8, and if positive were titrated. A serum was regarded as positive when it showed in the test at least 75 % fixation. Any specimen showing less than this reaction was considered doubtful and was retested at 1 in 4 dilution. Only if it then gave 75–100 % fixation was it regarded as positive.

RESULTS

Blood specimens were obtained from individuals of various ages as shown in Table 1. The children under 10 years old were in-patients mostly with minor illnesses at three children's general hospitals; blood was taken on admission or shortly afterwards. The 10- to 15-year-olds included seventy-five children from the same hospitals and from an orthopaedic hospital, and 182 healthy children from two mixed day schools. The older age groups comprised healthy persons who had blood taken for tests needed for travel. Socio-economic status was not investigated. The table illustrates the incidence of c.f. antibodies in the different age groups, using the 'Kerr' strain of virus as antigen.

Table 1. *Incidence of cytomegalovirus complement-fixing antibodies*

Age groups (years)	No. positive / No. tested	% positive
0–$\frac{1}{2}$	3/9	33
$\frac{1}{2}$– 5	4/93	4
5–10	15/97	15
10–15	54/257	21
15–25	47/130	36
25–35	62/114	54
35–75	46/85	54

The positive results obtained in infants under 6 months old are probably due to maternal antibody since, although the numbers are small, the incidence is closely similar to that of the adult child-bearing group. During the pre-school period, 6 months to 5 years, infection is apparently infrequent with only 4 % of children possessing antibodies. After 5 years antibodies become increasingly prevalent with age to reach a maximum incidence of 54 % by 25–35 years of age. This level is then

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maintained in the older age groups. No significant sex differences were noted. Table 2 shows the antibody titres of the positive sera and the geometric mean titres for each group.

The majority of the subjects in the above study were obtained at random from the general population, and included two groups of 10- to 15-year-old children attending two separate day schools. These were later compared with boys of the same age from a boarding school in which the opportunities for cross-infection are presumably greater (the latter were not included in the totals described in Table 1). Table 3 shows the strikingly higher incidence of antibodies in the boarding school population.

Table 2. *Distribution of titres of sera possessing complement-fixing antibodies*

Age group (years)	No. tested	No. of sera having indicated titre						Geometric mean titre
		8	16	32	64	128	256 +	
½– 5	4	—	—	2	—	—	2	90
5–10	12	2	—	—	3	—	7	97
10–15	53	2	1	24	6	18	2	56
15–25	47	8	—	21	9	4	5	40
25–35	62	12	—	44	—	6	—	28
35–75	46	9	—	18	6	3	10	46

Table 3. *Incidence of complement-fixing antibodies in three school populations*

School	No. positive / No. tested	% positive
Day 1	35/121	29
Day 2	15/83	18
Boarding	40/50	80

Specificity of the complement-fixation test

Cross-c.f. tests were carried out using seven strains of cytomegalovirus. The three American strains, 'Kerr', 'Davis' and 'Ad 169', have been shown to represent three distinct serotypes on the basis of the neutralization test (Weller, Hanshaw & Scott, 1960). The London strains were 'Aravi' isolated from a 6-month-old child with a malignant giant haemangioma, 'Sh' isolated from an apparently healthy 7-day-old baby who was found to have an enlarged liver and spleen and a reduced platelet count, and '138' and 'Rawles' isolated respectively from a fatal case of neonatal cytomegalic inclusion disease and from a 4-year-old boy with symptomless hepatomegaly and abnormal liver function tests (Stern *et al.* 1963; Stern & Tucker, unpublished). Since it has not yet proved possible to prepare animal antisera against these strains of virus they were tested against human sera from cases of current or recent infection. These were available from the three infants from whom strains 'Aravi', 'Sh' and 'Rawles' were isolated. Sera were also obtained from their mothers, who were found at the time not to be excreting virus, and from a 4- and a 2-year-old sibling of the 'Aravi' and 'Sh' cases respectively. The former was excreting virus without symptoms. Virus was also isolated

from the other child, who was found to have grossly abnormal liver function tests but was clinically well. An additional serum tested was from a woman (Ogg.) who had given birth to a fatal, histologically confirmed case of neonatal disease 1 year previously. The C.F. antibody titres of these various sera as determined with antigens prepared from each of the seven virus strains are shown in Table 4. Each serum cross-reacted with all seven viruses, and no evidence was obtained of significant antigenic differences among these strains.

Table 4. *Complement-fixation tests with sera from recent infections and seven strains of cytomegalovirus*

	Antigen						
Serum	Kerr	Ad 169	Davis	Aravi	138	Sh	Rawles
Aravi	128*	128	64	128	32	64	256
mother	64	64	64	128	64	64	256
sibling	64	64	64	64	64	64	128
Sh	32	32	16	8	8	16	32
mother	64	64	64	16	16	64	128
Rawles	32	128	32	16	16	32	64
mother	128	64	32	16	8	8	64
sibling	32	32	32	8	8	16	32
Ogg.	64	64	64	64	64	64	128

* Serum titre.

A number of positive and negative sera from the normal population study were re-examined, at 1 in 8 dilution, in a single C.F. test against the seven strains of viruses. These comprised twenty-five negative sera from each of the 5–10, 10–15 and 25–35-year-old age groups, and 15 positive sera each from the 5–10 and 25–35-year groups. Previously 100 negative sera and 40 positive sera from 5–15-year-old children in an institution (Stern & Elek, unpublished) had been examined simultaneously with strains 'Kerr', 'Ad 169' and 'Davis'. With every serum in these tests identical positive or negative results were obtained with all the virus strains used.

These various findings would appear to indicate that the seven strains of cytomegalovirus, isolated from the widely separated areas of London and the United States, have common group-specific C.F. antigens. Although many more strains require to be examined before it can be concluded that all cytomegaloviruses possess a common group antigen, the use of a single strain in an epidemiological survey seems to be justified.

Serum reactions with control antigen prepared from uninoculated human embryonic fibroblasts have been observed only three times in well over 1500 serum tests. One serum was from a 2-year-old mentally retarded child from a serological study of cytomegalovirus infection in relation to mental deficiency (Stern & Elek, unpublished). The other two sera were both from women who had recently given birth to infants with neonatal hepatitis. Cytomegaloviruses were not isolated from the urine or throats of the infants and a specific diagnosis could not be made in either case.

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DISCUSSION

In many virus infections the c.f. antibody response is transient, while neutralizing antibodies persist for many years. However, in other infections such as those caused by the herpes viruses c.f. antibodies persist for life, and can therefore be used for an epidemiological survey of past infection. This also applies to the closely related cytomegaloviruses (Rowe *et al.* 1956).

Infection with cytomegaloviruses, as opposed to clinical disease, is obviously widespread in London. The incidence, based on our serological findings, appears to be low in early childhood since only 4 % of pre-school children have demonstrable antibodies. This agrees with the infrequent finding of cytomegalic cells in the salivary glands of unselected paediatric autopsies in this country (Baar, 1955; McDonald, personal communication). The incidence of infection mounts steadily through the school-age period and adult life to reach its maximum of 54 % by 25–35 years. Young adults are, therefore, apparently exposed as much to infection as school-children. It is probable that children are the main source of infection since it has been shown that they may excrete virus in their mouths, as well as in the urine, for prolonged periods (Rowe, Hartley, Cramblett & Mastrota, 1958). There is little information concerning the excretion of virus in infected adults, although it has been demonstrated that women who have given birth to babies with cytomegalic inclusion disease may continue to excrete virus in their saliva for many months without symptoms (Medearis, 1964). However, the importance of adult carriers in the spread of infection is as yet unknown. The fact that the incidence of c.f. antibodies does not increase after the age of 35 suggests that older individuals are less exposed to infection, perhaps because there is less intimate contact with small children.

The persistence of c.f. antibodies in the population suggests, by analogy with herpes simplex, that after recovery from the primary infection the virus is not eliminated from the body but persists in a latent form. Activation of such latent infection occurring as a complication of diseases which depress the immunity mechanisms of the body has been invoked to explain the rare disseminated disease in adults (Nelson & Wyatt, 1959); although it is also possible that this follows exogenous infection and that dissemination results from the failure of immunity. At present there is no evidence on whether intermittent reactivation with excretion of virus occurs in healthy individuals, as it does in herpes simplex.

The low rate of infection among school-children indicates that the virus does not spread easily and that close and prolonged contact may be necessary for cross-infection. Thus, among 83 children attending a day school only 18 % had cytomegalovirus antibodies as compared with a 45 % incidence of herpes antibodies. The increased rate of infection with closer prolonged contact is shown by the 80 % incidence of cytomegalovirus antibodies in the boarding school, and high infection rates have also been demonstrated among institutional children and family contacts (Hanshaw & Simon, 1962; Weller & Hanshaw, 1962; Stern *et al.* 1963). Cytomegalovirus is more labile in the extracellular environment than the herpes virus and this probably accounts for the low degree of contagion.

Our findings at all ages differ considerably from those of Rowe and his colleagues

(1956) in Washington, who found C.F. antibodies in 30 % of 5-year-olds and 81 % of adults over 35 years. It seemed possible that our lower figures were due to the use of a heterotypic strain of virus whereas Rowe *et al.* employed a strain (Ad 169) isolated in the Washington area. However, the re-examination of sera with antigens prepared from various viruses isolated in the United States, and locally in London, shows that this is not the explanation. Our finding of common group-specific C.F. antigens is in agreement with that of Medearis (1964), who also showed that although the early C.F. antibody response in neonatal disease may tend to be strain specific it later becomes group specific. The more likely explanation of the differences in antibody prevalence between London and Washington is to be found in the socio-economic status of the two populations tested. Differences in antibody incidence have previously been demonstrated in small children of various countries, ranging from 15 % in Virginia to 85 % in Egypt (Rowe, 1960). In the same way, cytomegalic cells have been found in the salivary glands of 10–12 % of unselected paediatric autopsies in Europe and North America (Farber & Wolbach, 1932; McCordock & Smith, 1934; Seifert & Oehme, 1957) as compared with 18 % and 32 % in the less highly developed areas of South America and Indonesia (Potenza, 1954; Prawirohardjo, 1938). As with poliovirus infections, improved standards of hygiene reduce the chances of infection in early childhood so that the first striking increase of infection occurs in older children when they come together at school.

In view of the finding that about a third of women become infected and acquire their antibodies during the main child-bearing period (15–35 years of age) it is surprising that neonatal disease is apparently so rare in Britain as compared with other countries. It may in fact be more common than previous reports would indicate. The severe neonatal syndrome is probably often misdiagnosed during life because of unfamiliarity with the disease, and even in fatal cases diagnosis may be possible only by virological methods (Weller & Hanshaw, 1962; Stern *et al.* 1963). In addition, many non-fatal cases undoubtedly occur without the full classical neonatal picture. They may present with only mild hepatosplenomegaly; such cases can be recognized only by means of virus isolation.

After the neonatal period the bulk of infection is apparently subclinical. A proportion of the cases, however, may possibly be associated with minor illness. Women who have produced babies with neonatal disease have sometimes given histories of respiratory illnesses during the course of pregnancy (Weller & Hanshaw, 1962; Medearis, 1964), and the almost invariable presence of lung lesions in both neonatal and adult disease stresses the importance of the respiratory site of entry of the virus. Small children with apparently symptomless excretion of cytomegalovirus in the urine may have hepatomegaly or abnormal liver function tests (Rowe *et al.* 1958; Hanshaw & Simon, 1962). Whether this is always transient or occasionally results in more chronic liver disease is as yet unknown. Disease caused by cytomegaloviruses is clearly more prevalent and has a broader range of symptomatology than previously recognized. A clear picture can only emerge of the lesser forms of illness in children and adults if the disease is kept in mind and virological investigations are used more extensively.

SUMMARY

A serological study of cytomegalovirus infection in London shows it to be prevalent. Under 5 years of age only 4 % of children have antibodies, but this increases to 15 % by 10 years of age and 21 % by 15 years. The maximum incidence of 54 % is reached by 25–35 years, and this is maintained in the older age groups of the population. In two mixed day schools the incidence of infection was significantly lower than in a boarding school, suggesting that close prolonged contact is required for spread of infection.

Different strains of cytomegalovirus possess common group-specific complement-fixing antigens.

It is suggested that clinical disease caused by cytomegaloviruses is more frequent and more varied than previously believed, although diagnosis may be difficult without virological help.

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