INFECTIOUS MONONUCLEOSIS: DETECTION OF HERPESLIKE VIRUS AND RETICULAR AGGREGATES OF SMALL CYTOPLASMIC PARTICLES IN CONTINUOUS LYMPHOID CELL LINES DERIVED FROM PERIPHERAL BLOOD

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Although an etiologic agent has not been isolated and identified, infectious mononucleosis has long been suspected to be an infectious disease, presumably of viral etiology. This concept is further supported by certain epidemiological features of the disease and by the nature of the acute illness. Recently, cytomegalovirus,[1] myxovirus,[2] and the herpeslike virus (HLV)[3] have all been implicated in the pathogenesis of this disease in man. In ultrastructural studies of circulating atypical lymphocytes from patients with infectious mononucleosis[4] and of lymphoblastoid cells derived from bone marrow cultures of infectious mononucleosis patients,[5] viral particles could not be demonstrated.

Recent reports from several laboratories have revealed that circulating cells from the peripheral blood of patients with infectious mononucleosis have the potential for long-term proliferation in vitro.[6] In this laboratory, 16 suspension cultures from nine patients with heterophile-positive infectious mononucleosis have been obtained in continuous culture. These established cell lines have been shown to synthesize immunoglobulin,[7] to produce interferon,[8] and to have chromosomal abnormalities similar to those consistently found in cultured cells derived from Burkitt's lymphoma.[9] In the course of studying the fine structural features of these cell lines, viruslike particles have been detected and identified. In the present communication the results of these findings will be reported.

Materials and Methods.—Sixteen continuous suspension cultures were established from nine patients with heterophile-positive infectious mononucleosis (Table 1) by means of techniques which have been described previously.[6] Samples of cell suspensions were examined periodically for the presence of microbial agents.[10] In each instance, cell lines were found to be free of detectable viruses, bacteria, fungi, mycoplasma, and endotoxins. From nine patients with infectious mononucleosis, 14 cultures were processed for electron microscopy (Table 2). As controls, other continuous cultures from peripheral blood were processed collaterally—one from a patient with lymphoblastic leukemia and another from a patient with leukocytosis of unknown etiology. In addition, thoracic duct and peripheral blood cultures from a patient with hyperglobulinemia of unknown etiology, buffy coats from the peripheral blood of six patients with infectious mononucleosis, and those from three clinically healthy individuals were also examined. Four of the contin-

TABLE 1. *Comparative summary of attempts to establish continuous cell lines from peripheral blood.*

	Cultures Initiated		Continuous Suspension Cultures Established	
	No. of individuals	No. of specimens	No. of individuals	No. of specimens
No obvious clinical illness	18	24	0	0
Infectious mononucleosis	23	66	9	16

uous cell lines (33H, 33J, 42D, 42F) were irradiated with a 250-kv Quadrocondex dual-tube apparatus with a dose range of 1500–9000 r and were examined 10 days after irradiation. After centrifugation, cell buttons were fixed with glutaraldehyde,[12] postfixed with osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in araldite or epon. Thin sections were stained with uranyl acetate and lead citrate. Negative-stain preparations of four cultures (33H, 33J, 42D, 42F) were examined. Concentrated suspensions of frozen-thawed cells were stained with 1% potassium phosphotungstate.

Results.—In approximately 1–5 per cent of cell cross sections, reticular arrays of tubules or particles 22 mμ in diameter were present in the cytoplasm (Figs. 1 and 2). These aggregates of particles generally occurred in cells which had relatively large quantities of rough endoplasmic reticulum (RER) and were bound by membrane which was often continuous with the RER membranes. The particles thus appeared to be within endoplasmic cisternae and to be interconnected to form an irregular reticular arrangement (Fig. 2). Crystalloid arrays were rarely found. At high magnifications, a bileaflet, unit membranelike structure enclosed material of medium density (Fig. 3). The particle appeared to bud from the inner surface of the ER membranes, and there seemed to be a continuity between the outer bileaflet and the ER membrane. The aggregates of particles were found in intact cells which showed little or no morphologic evidence of damage or degeneration.

These peculiar cytoplasmic structures had a very consistent and characteristic appearance and were present in all 14 cultures derived from patients with infectious mononucleosis. Similar particles were not observed in the culture from the patient with lymphoblastic leukemia, but the culture from the patient with leukocytosis of unknown etiology did show arrays of particles morphologically identical to those found consistently in the infectious mononucleosis cultures. The thoracic duct culture from the patient with hyperglobulinemia did not show the cytoplasmic particles, but the peripheral blood culture from the same patient showed the cytoplasmic structures as well as the herpeslike particles.

Typical herpeslike particles (Fig. 4) were also noted in four of the 14 infectious mononucleosis cell lines examined (Table 2). They occurred in nuclei of degenerative cells (Fig. 4) or in debris of dead cells. Of the 16 cell lines examined by the direct fluorescent antibody technique, 12 lines were reactive. Positive immunofluorescence staining was detected in 0.1–1.0 per cent of the cell population in these 12 lines (Table 2). As seen in Table 2, herpes virus could not be

FIG. 1.—Cell containing many dilated RER cisternae filled with granular proteinaceous material and having an appearance much like that of a plasma cell. Note the aggregates of small dense particles below the nucleus. PGLC 55B. ×17,000.

FIG. 2.—Aggregates of particles 22 mμ in diameter which appear to be within RER cisternae. The particles have a less dense center and often appear interconnected forming a reticulum. Aggregates of particles such as this were noted in a relatively high percentage of cells in all 14 cell lines derived from peripheral blood leukocytes of patients with infectious mononucleosis. PGLC 50B. ×53,000.

FIG 3.—Higher magnification of the RER-associated particles which appear to be composed of a unit membranelike structure enclosing material of less density. Occasional particles appeared to bud from the inner surface of the ER membrane. Elongated rod-shaped or tubular forms were present usually in aggregates close to the nucleus. A ribosome attached to the outer surface of the RER membrane is at upper right of the micrograph. PGLC 51E. ×200,000.

detected in eight of the cell lines which displayed positive immunofluorescence. It is of interest that in the cell line (42D) which did not display immunofluorescence, herpeslike particles were detected only after irradiation of the culture. The nuclear particles ranged from 90 to 100 mμ in diameter with a 50–60-mμ

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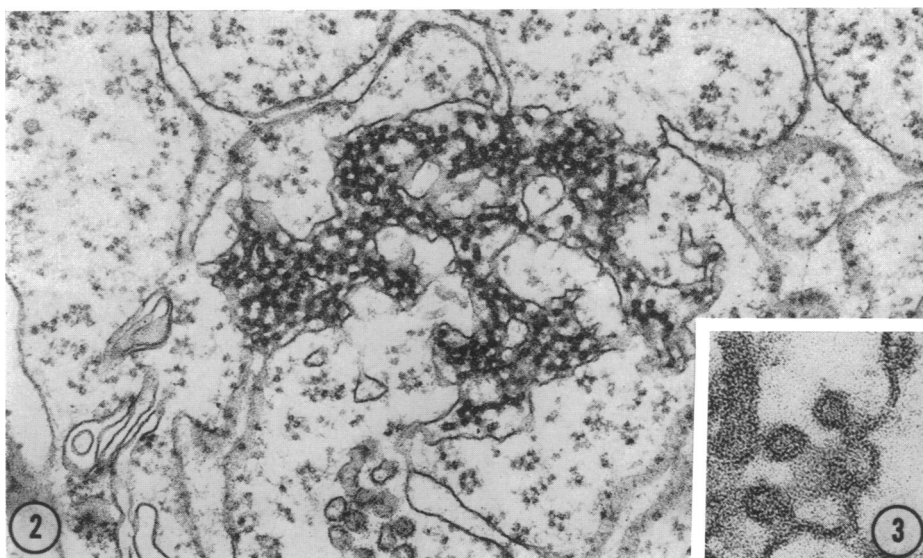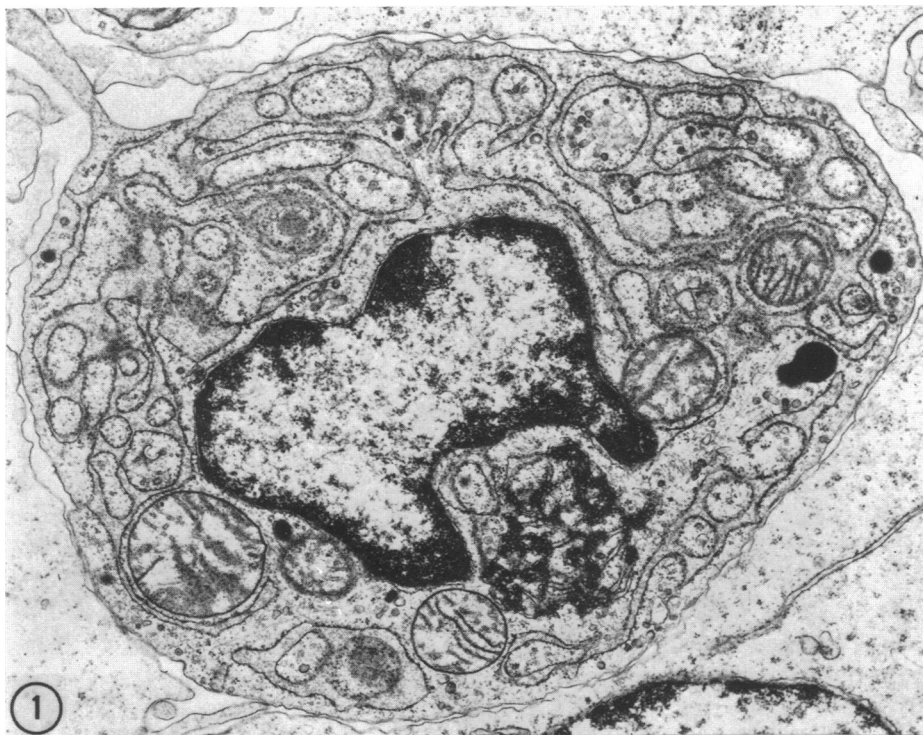
nucleoid. The herpeslike particle and the RER-associated small particle were observed in the same cell on only one occasion, but the two types of particles were frequently present in adjacent cells.

Extracellular herpeslike particles usually had an additional surrounding membrane and a total diameter ranging up to 160 mμ (Fig. 5). Much smaller

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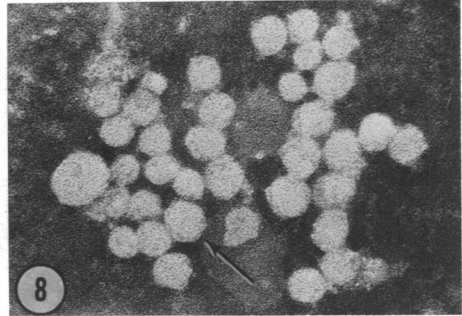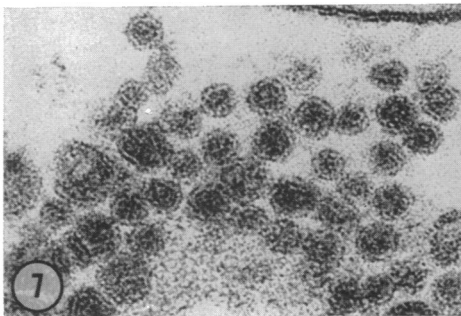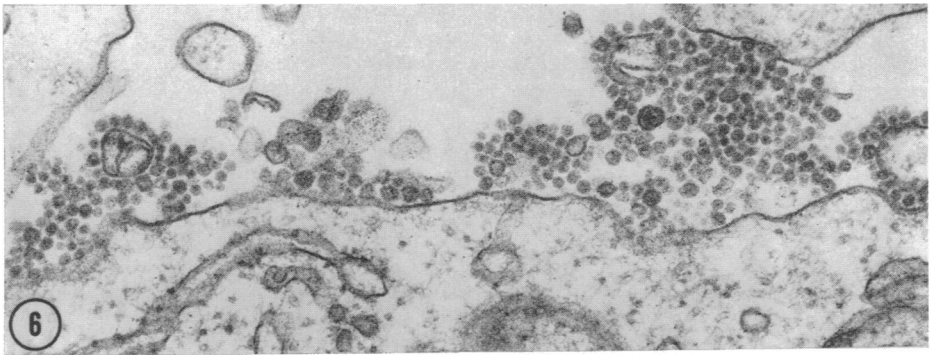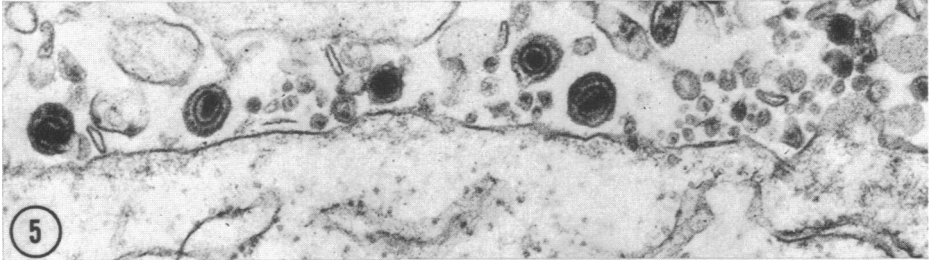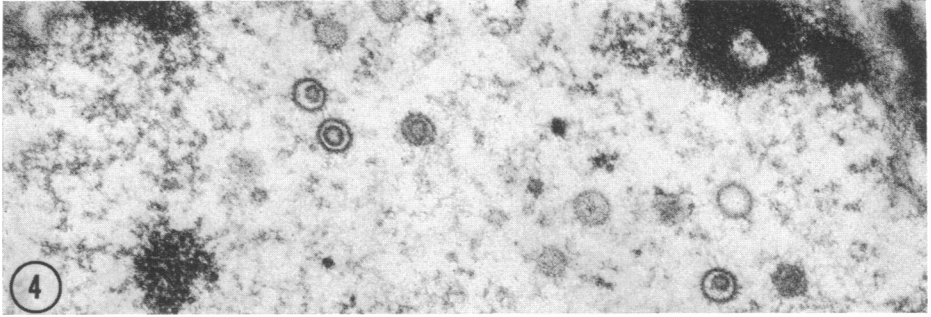
extracellular particles of relatively uniform size (25–50 mμ) (Figs. 5–7) were often seen in close proximity to herpeslike particles. A small central density was present in many of these small particles, whereas others seemed to be almost completely filled with medium-dense material. The periphery of the particles often appeared as a unit membranelike structure (Fig. 7). The cells with which the small extracellular particles were associated generally showed few degenerative changes and rarely contained the herpeslike particle. Large aggregates of small particles such as those illustrated in Figures 6 and 7 were, however, noted only in cell cultures which were also found to contain the herpeslike particle.

Negative stain preparations from four cell lines revealed aggregates and isolated particles which ranged from 30 to 40 mμ in diameter (Fig. 8). These particles appeared at times to have an outer membrane and were similar in size to the small extracellular particles noted in thin sections (Fig. 7). Capsomeres were not observed. Herpeslike particles were not identified in negative stain preparations.

The appearance of peripheral blood leukocytes from patients with infectious mononucleosis was essentially the same as that previously described.[4] Neither herpeslike virus particles nor cytoplasmic aggregates of small particles were detected in the peripheral blood leukocytes.

Discussion.—Since 1964, when Epstein *et al.*[13] first detected the presence of viruslike particles in continuous lymphoid cell lines derived from Burkitt's lymphoma, the so-called herpeslike virus has received a great deal of attention. These particles have been found in cell cultures derived from the peripheral blood of patients with leukemia,[14] from patients with a variety of other malignant diseases,[15] and from apparently healthy individuals.[16] The HLV particles are antigenically distinct from known herpes viruses. In addition, attempts to demonstrate biologic activity with the HLV have generally met with difficulty because these particles usually do not infect cell lines susceptible to members of the herpes-virus group.[15] Recently, Stewart *et al.*[18] reported that cell-free concentrates containing the HLV particles can infect newborn thymectomized hamsters when inoculated intracerebrally, and transmission of the particles to human leukocyte cultures has also been reported.[19]

Using an indirect immunofluorescent technique, Henle *et al.*[3] recently showed

Fig. 4.—Incomplete herpeslike particles located within a nucleus. The particles (100 mμ in diameter) have empty-appearing nucleoids measuring 50–60 mμ. Such nuclear particles were noted only in cells showing severe degenerative changes or in dead cells. PGLC 42D, 3000 r. ×50,000.

Fig. 5.—Extracellular herpeslike particles which have the same dimensions as the nuclear particles but which appear surrounded by an additional membrane coat giving an over-all diameter ranging up to 160 mμ. These "complete" forms have very dense nucleoids. Note also the small particles (25–40 mμ in diameter) composed of a single outer membrane and a central density. PGLC 55B. ×50,000.

Fig. 6.—Clusters of extracellular small particles (25–40 mμ), some of which appear to bud from the cell surface. PGLC 55B. ×50,000.

Fig. 7.—High magnification of the small extracellular particles which are composed of an outer membrane and central dense material. PGLC 50B. ×200,000.

Fig. 8.—Negative-stain preparation with neutralized 1.0% phosphotungstic acid showing aggregates of particles measuring 25–40 mμ in diameter. Occasional particles appeared to show an outer membrane (*arrow*). PGLC 33H. ×200,000.

TABLE 2.   *Summary of the findings on the established infectious mononucleosis cell lines.*

Established cell line	Days after onset of clinical illness	Proliferation evident (days in culture)	Cells fixed for electron microscopy (days in culture)	Viral antigen detected by immuno-fluorescence*	Herpeslike virus detected by electron microscopy	Presence of cytoplasmic reticulum of 22-mμ particles
PGLC 33H	44	36	64,192	+	−	+
PGLC 33J	86	21	119	−	−	+
PGLC 42D	9	44	79,105	−	+†‡	+
PGLC 42F	15	41	76,111	−	−	+
PGLC 44B	10	35	117	+	−	+
PGLC 50B	9	24	77	+	−	+
PGLC 50D	16	40	70	+	−	+
PGLC 51D	45	60	115	+	−	+
PGLC 51E	112	63	85	+	+‡	+
PGLC 53B	22	49	108	−	−	+
PGLC 53D	29	46	102	+	−	+
PGLC 54B	12	53	123	+	+	+
PGLC 55B	20	34	46	+	+‡	+
PGLC 55E	63	...	N. E.	+	N. E.	N. E.
PGLC 55F	68	...	N. E.	+	N. E.	N. E.
PGLC 56B	18	74	123	+	--	+

N. E., not examined.

* Directed immunofluorescent staining was performed with a highly reactive anti-HLV serum.[11] In the positive cell lines, 0.1–1.0% of cells examined were immunofluorescent.

† The herpeslike virus particles were detected only after X irradiation (3000 r).

‡ Large aggregates of extracellular viruslike particles (25–40 mμ) were present (see Figs. 6 and 7). These particles were morphologically distinct from the herpeslike virus.

that antibodies to the HLV developed in patients with infectious mononucleosis and concluded that this virus is the probable etiologic agent in infectious mononucleosis. In the present study, with a highly reactive fluorescein isothiocyanate-conjugated anti-HLV serum, positive immunofluorescence was demonstrated in 12 of the 16 infectious mononucleosis cell lines examined. It is therefore not surprising that HLV particles were detected by electron microscopy. The virus particles and the cellular alterations in cells containing the HLV particles were identical to those reported in cultured cells from Burkitt's lymphoma[13] and from patients with leukemia.[14] The frequency of occurrence of HLV particles in the infectious mononucleosis cell lines, however, appears to be no higher than in cultures from apparently normal individuals[16] and from patients with leukemia, lymphomas, and other malignancies.[14, 15, 17]

A far more consistent finding in the cell cultures derived from patients with infectious mononucleosis was the presence of reticular arrays of 22-mμ particles in the RER cisternae. These structures were present in a relatively high percentage of cells in all 14 cell lines studied. These particles were also observed in two other cell lines—one from a patient with idiopathic leukocytosis and another from a patient with hyperglobulinemia of unknown cause. Although the exact nature of these particles awaits further definition, there is evidence that they are viral-associated. Apparently identical structures often with a more orderly crystalloid arrangement than was present in the infectious mononucleosis cell lines have been described in tumor cells induced in monkeys by Rous sarcoma virus (RSV),[20] meningiomas and meningeal sarcomas induced in dogs by RSV,[21]

cultured monkey kidney cells infected with rubella virus,[22] and in two lymphoma-derived cell lines—one from a Burkitt's tumor and a second from an American lymphoma resembling Burkitt's tumor.[23] In recent unpublished studies, Zeve[24] observed the presence of irregular and crystalloid arrays of RER-associated small particles in two cultures from Burkitt's lymphoma and in a chimpanzee which developed acute myelogenous leukemia two years after receiving total body irradiation. In the latter, the arrays of small particles were present in cells of many major organs and in peripheral blood and persisted in cell cultures established from peripheral blood and other tissues.[24] The precise relationship of these structures to virus infection or replication has not been demonstrated. The available evidence, as well as the findings in the present study, suggests that these cytoplasmic aggregates of 22-mμ particles may play a role in states of abnormal cellular proliferation *in vivo* and *in vitro*. The reticular arrays of cytoplasmic particles may represent cellular alterations occurring secondary to HLV infection. On the other hand, these cytoplasmic structures may be indicative of infection by a second virus unrelated to HLV. This host cell-virus interaction may then be responsible for the cellular proliferation which occurs with the clinical appearance of infectious mononucleosis and the long-term proliferation of the continuous cell lines. Since RER-associated particles have been noted previously in mammalian tumors induced by an RNA tumor virus,[20, 21] a virus of this type would appear to be a likely etiologic agent in infectious mononucleosis. Another interpretation of our data is that a dual infection may exist in the continuous cell lines derived from patients with infectious mononucleosis. Of interest in this regard is the observation that the small extracellular particles were far more numerous in cell cultures which also contained the herpeslike particles. Thus, infection with a defective virus which requires a helper virus for production of infective particles could account for the difficulties encountered in the experimental transmission of infectious material both *in vitro* and *in vivo*. In support of this, the concept of defective virus infection in human disease has recently been introduced by the demonstration in man of antibodies to adenovirus-associated virus. This virus is defective, since the presence of adenovirus is required for production of infective particles *in vitro*.[25] It is thus possible that the rise in antibody to HLV in patients with infectious mononucleosis may result from similar interactions.

The relationship of the cytoplasmic particles and small extracellular particles to HLV, however, remains to be established, as does the relationship of the herpeslike particles or other apparent virus particles to the pathogenesis of infectious mononucleosis.

Summary.—Fourteen continuous suspension cultures derived from peripheral blood leukocytes of nine patients with heterophile-positive infectious mononucleosis were examined by electron microscopy. The Burkitt's tumor-associated, herpeslike virus was identified in four of the 14 cell lines. All 14 of the cultures contained, in a relatively high percentage of cells, reticular arrays of small (22-mμ) particles in association with the rough endoplasmic reticulum. These aggregates of small particles were also found in two other continuous cell lines—one from a patient with leukocytosis of unknown cause and another from a

patient with idiopathic hyperglobulinemia. The relationship of the small cytoplasmic particles to infective viral particles has not been established, but they appear to be virus-associated.

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