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☐ Original Article ☐

Hairy Cell Transformation of Human Peripheral Blood Lymphocytes by *Coxiella burnetii*

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Hairy cell (HC) transformation of human peripheral blood lymphocytes (PBL) by Coxiella burnetii was studied to clarify the significance of persistency of C. burnetii in a hairy cell line (designated "TOL"). TOL cells which exhibited HC characteristics in hairy cell leukemia (HCL) were persistently infected with C. burnetii. Two strains of C. burnetii, our isolate from TOL cells and the original isolate in 1935, the Nine Mile strain from American Type Culture Collection (ATCC, U.S.A), were inoculated to PBL cultures. HC transformation not only by our isolates (87%) but also by Nine Mile strain (100%) was demonstrated in an average of 20 days. The original observation that Coxiella induced HC transformation in vitro was also confirmed in experiments with PBL exposed to C. burnetii in vivo. Spontaneous development of HC were observed in cultures of PBL only from coxiellemic cases (12/24) but not from C. burnetii negative cases (0/57). All HC cell lines (34) as determined by their morphology and cytochemical markers of HC in HCL remained infected with C. burnetii invariably.

Key Words: *Coxiella burnetii*, cytoplasmic granule, cytoplasmic projection, hairy cell, hairy cell leukemia, Immortalization, peripheral blood lymphocytes, tartrate resistant acid phosphatase, surface immunoglobulin

A hairy cell line, designated TOL, was established in cultures of PBL from a Korean farmer whose blood was received for an AIDS test. In addition to the hairy morphology(Schrek & Donnelley, 1966), TOL cells demonstrated two other characteristics associated with HC described in HCL; the presence of tartrate resistant acid phosphatase (TRAP) in the cytoplasm and of surface immunoglobulins (Sig) in the membrane. HCL is a well-defined neoplastic disease marked by the abnormal expansion of HC which often produce monoclonal antibodies. There have been reports of Epstein-Barr viruses (EBV) in sera and HC in HCL patients (Sairenji *et al.* 1984; Wolf *et al.* 1990), a report of human T-cell leukemia virus type II (HTLV-II) from T-cell variants of HCL (Kalyanaraman *et al.* 1982), and of the association of HCL with chronic exposure to benzene (Askoy 1987; Ng *et al.* 1987; Mc-Kinney *et al.* 1988). However, the etiology and pathogenesis of HCL have not been determined. When TOL cells were viewed by transmission electron microscopy and examined by immunofluorescence assay (IFA), it was found that the cells of each generation were parasitized by *C. burnetii*, the agent of Q fever. The persistency of *C. burnetii* in the established HC cell line offered a system in which to explore the question of whether the *Rickettsia* could induce transformation of normal lymphocytes to HC.

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MATERIALS AND METHODS

Preparation and establishment of PBL lines

Fresh PBL from cases who were sent for human immunodeficiency virus (HIV) testing had been routinely cultured for 5 years. PBL

separated from plasma by Ficoll-Hypaque (Pharmacia, Upsala, Sweden) was explanted in plastic culture flasks (Costar, U.S.A.) containing minimum essential medium (MEM; Hazleton Biologics, U.S.A.), 15% fetal bovine serum (FBS; CSL, Australia), and penicillin and streptomycin (100 IU & 100 μg/ml). The cells were cultured at 37℃ in a humidified CO_2 incubator. Later, the medium, MEM, was supplemented with non-essential amino acid (Hazleton Biologics). Bacterial and chlamydial contamination of the cultures were checked by use of thioglycolate and microtests by use of McCoy cells (Yoder *et al.* 1981). TOL cell line was established in the BPL culture from a Korean male whose serum was negative for antibody to HIV. For some of the preliminary experiments, human cord blood lymphocytes (CBL) prepared by the same method were also used.

Eligibility criteria for HC

HC was determined by the living cell morphology and immuno-histochemical markers of the cells. Cells incubated in a microscopic chamber were examined by phase contrast microscopy (magnification, 1000x, oil immersion; Zeiss, ICM405, Germany) and laser scanning microscopy (Zeiss, LSM). The dynamic nature of the cytoplasmic projections were analyzed by videomicroscopy. The entire procedure for cell marker studies was done at 4℃. Cells suspended in Ca^{++} & Mg^{++}-free phosphate buffered saline (PBS; 1×10^5 cells/ml) were centrifuged at 13,000 g for 10 sec and precipitated cells were treated with 20 μl of the primary antibody specific to the antigen to be tested (monoclonal antibody to CD3, CD4, CD8 and Slg; Beckton Dickinson, U.S.A.) for 45 min.

The cells washed 3 times with Ca^{++} & Mg^{++}-free PBS were treated with fluorescence-labelled anti-mouse rabbit serum (KPL, U.S.A.) for 45 min. The cells were then washed once and a drop of the cells was placed on slides for fluorescent microscopy (Zeiss Epifluorescence, Short Arc Mercury HBO 50 lamp, BP450-490, FT510, LP520, Germany). Antigens of HIV, HTLV-I, and EBV in the cells were also determined by IFA with a specific antibody (IgG, DuPont, U.S.A.). The presence of TRAP in HC cells was verified following the method used by Yam *et al.* (Yam *et al.* 1971). IgG isotype in culture supernatant of HC transformed cells was determined by enzyme linked immunosorbent assay (ELISA) with monoclonal antibodies to Ig isotypes (Beckton Dickinson).

Identification of *C. burnetii*

The presence of intracellular organisms in the cells were examined by transmission electron microscopy (TEM; Phillips, CM-10, Netherlands) at 6,000 to 35,000×magnification. *C. burnetii* infection of the cells was determined by IFA. For IFA tests, the cells were collected on glass slides by cytocentrifugation, fixed in cold acetone for 15 minutes on microscopic slides. The cells were then treated with anti-*Coxiella* rabbit serum (from Pasteur Institute, Paris, France and/or ATCC). For controls, fresh normal PBL, negative reference serum, antiserum to *Rickettsia tustsugamushi* (ATCC), and the fluorescence labelled-antiserum were tested in various combinations.

Preparation of *C. burnetii* from TOL for inoculation

TOL cells were disrupted in the culture fluid with glass beads (0.5 mm in diameter; Biospec, U.S.A.) and centrifuged (Vetter, GMBH, Germany) at 400 g for one hour to precipitate cell debris. The supernatant was collected and centrifuged at 40,000 g (Beckman, U.S.A.) for one hour to concentrate organisms. The total amount of protein in the preparation was quantitated by Lowry's method. Stock solutions of the organisms were made by diluting the sediments in MEM to be one mg of protein per one ml of MEM and were frozen at −70℃ until use. *C. burnetii* in the stock solution was determined by the micro-immunofluorescence test described by Philip *et al.* (Philip *et al.* 1976). Although EBV antigen was not detected in TOL cell (as determined by IFA), the donor of the indicator TOL line had the antibody to EBV in his serum (as determined by ELISA). To eliminate EBV involvement in the hairy cell transformation, *C. burnetil* was prepared by passaging the organism from TOL into the monkey kidney cell line, Vero, which is not susceptible to EBV (Pope *et al.* 1968; Gerber *et al.* 1969; Pope *et al.* 1971) for one month.

Inoculation of *C. burnetii* isolated from TOL, Vero, and strain Nine Mile

The organisms isolated from TOL cells including the one passaged into Vero and the

Nine Mile strain of *C. burnetii* (the original strain isolated in 1935, ATCC) were tested for the ability to transform fresh PBL and cord blood lymphocyte (CBL) to HC. The frozen organisms were thawed rapidly, passed through 0.45 μm filters (Sartorius, Germany) and inoculated. A volume of 0.5 ml of inoculum in MEM were inoculated onto 5×10^6 of fresh PBL or CBL in a 25 cm^2 flask containing the growth medium. Each PBL culture received 100 μg of proteins of our isolate or the same volume of the Nine Mile strain of *C. burnetii* diluted in MEM.

Established cell lines

Established human lymphocyte lines of hu-

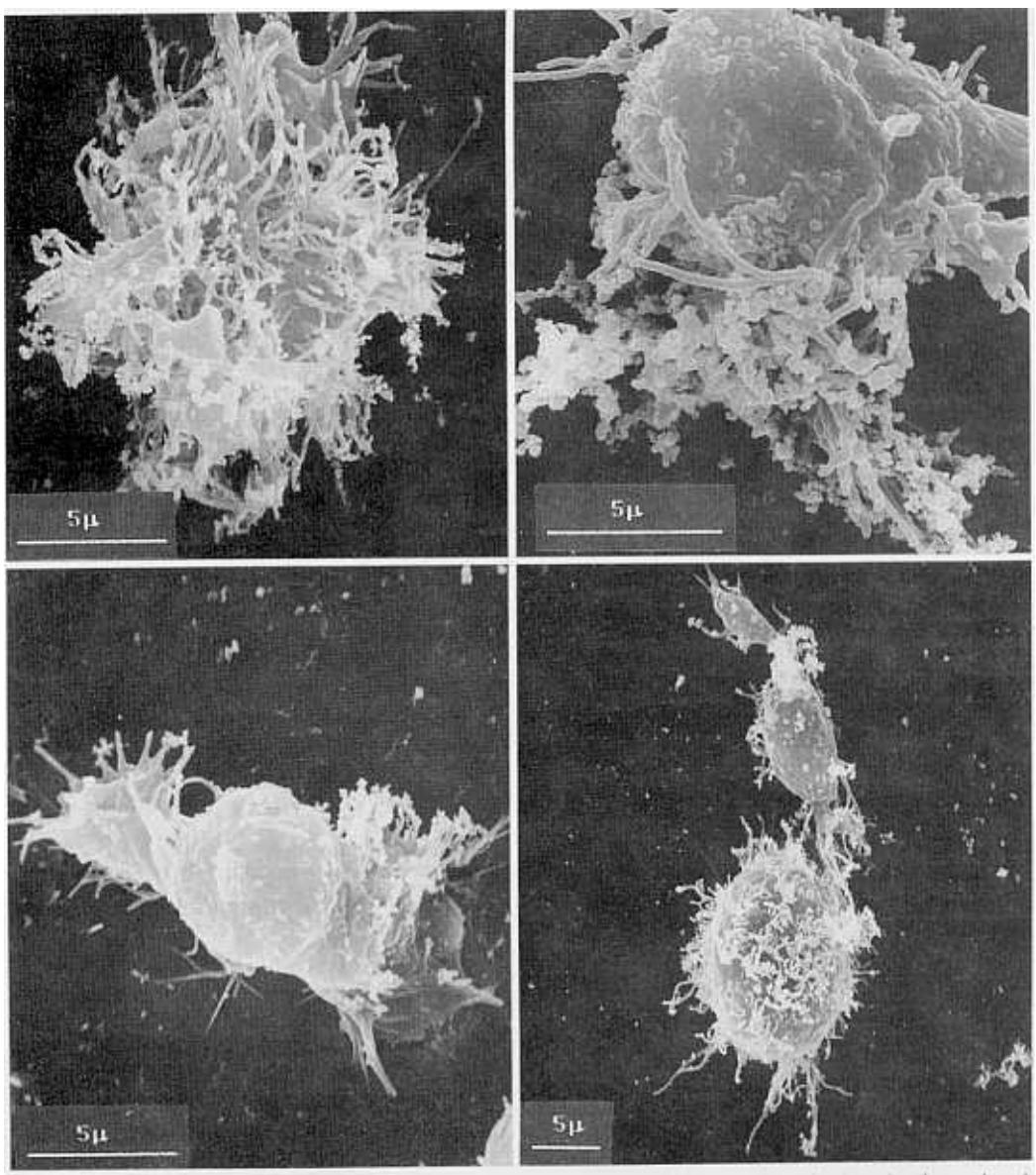

Fig. 1. *Scanning electron microphotographs of TOL cells with numerous filamentous projections in the entire circumference of the cell.*

man T-blast (MOLT-4), B-blasts (Raji, Daudi) and monkey kidney cells (Vero) were also inoculated following the same methods. These cell lines were originally purchased from ATCC, in 1983 and have been passaged in RPMI-1640 media for the lymphoid lines and MEM for Vero cells with 10% FBS and penicillin & streptomycin.

PBL from coxiellemic patients

Blood samples of 107 patients provisionally diagnosed as various hematologic disorders and normal volunteers were tested for *C. burnetii* infection and grouped by the test results. On delivery, a drop of the blood sample was directly smeared on the microscopic slides and tested for *Coxiella* infection in PBL by IFA assay. PBL were then separated and explanted in flasks for cultures by the method described earlier.

RESULTS

Characteristics of TOL cells

Initially, two lines of PBL with hairy mor-

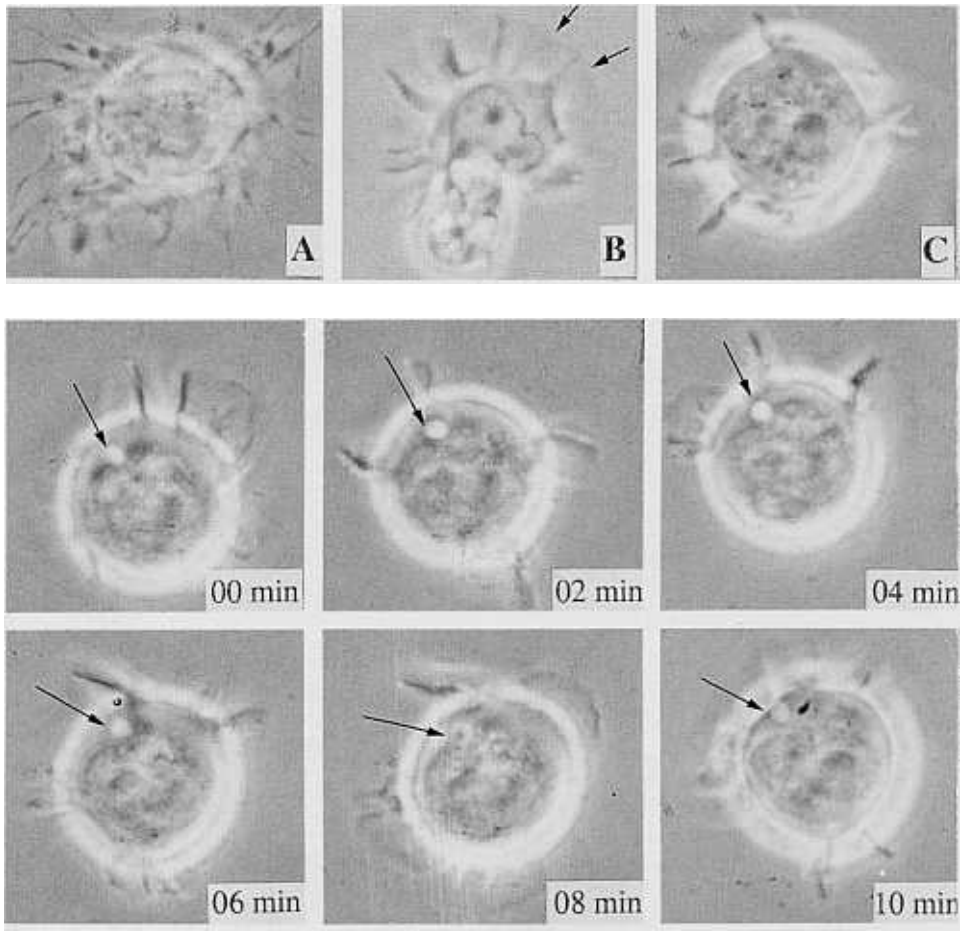

Fig. 2. Phase contrast microphotograph of living TOL cell in a special slide chambers, magnification 1000X. (A) Tol cell with numerous filamentous projections in the entire circumference of the cell. B) TOL cell with membrane ruffles (MAR) between cytoplasmic projections. (C) TOL cells with thick and branched extrusions. Time laps photograph (every two minutes) of pleomorphic presentation of a single hairy TOL cell in a special slide chambers, (magnification 1,000X). Note a vacuole(arrow) as a marker which expelled by the cells eventually.

phology had been maintained in culture. After a year of culture *in vitro*, only a line, designated TOL, became an established cell line. TOL line was established from a 27-year old Korean male who was pancytopenic. Most of the cells in TOL line exhibited numerous cytoplasmic projections and they adhered to each other in clumps floating in the culture medium (Fig. 1). These projections resembled those of HC originally described in HCL. The projections were

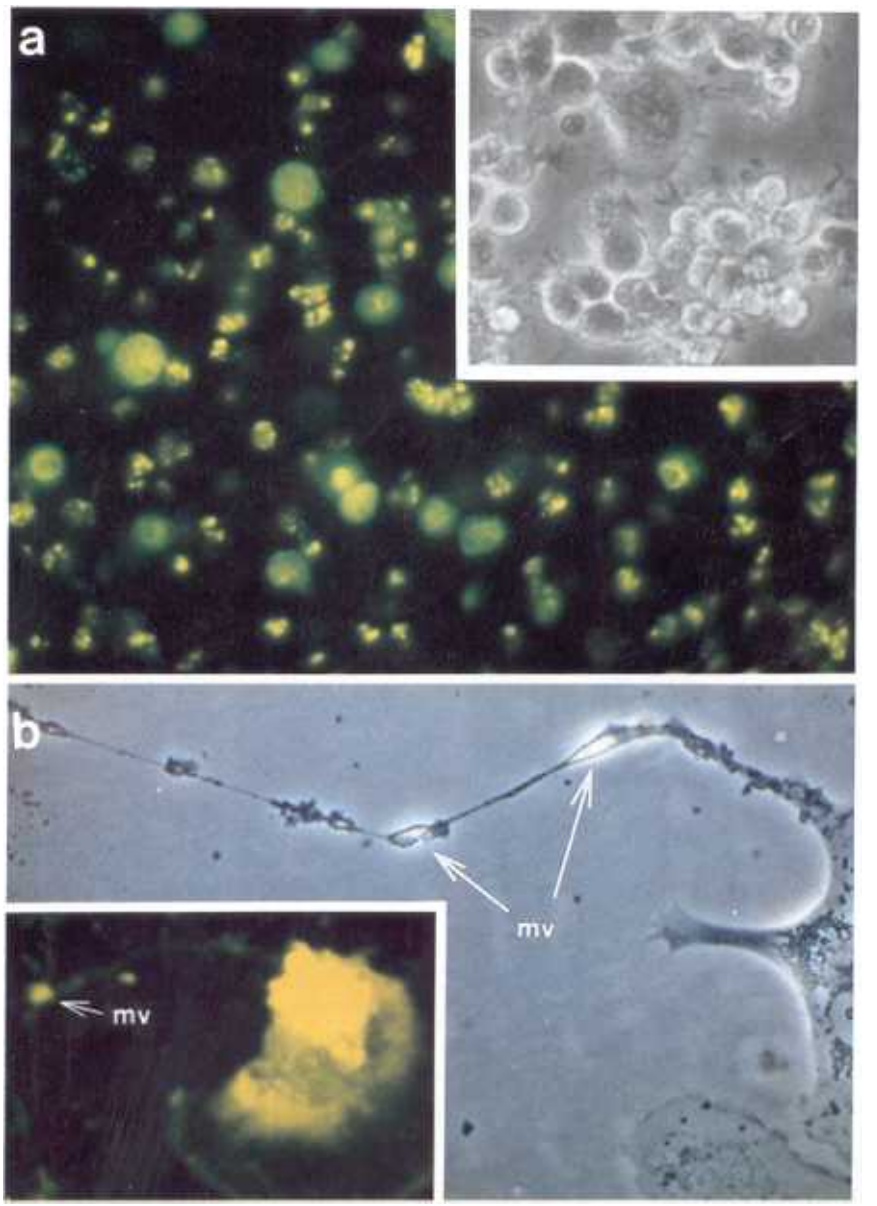

Fig. 3. *Innunofluorescence micrograph of TOL cells reacted with C. burnetii-specific rabbit antisea. (A) cell with cytoplasmic positive granules reacted with the anti-serum, 160Xand phase contrast photograph of TOL cells in various shape in culture (inset), 320X. (B) Phase contrast photograph of a TOL cell hairy projection with micro vacuoles (MV) which specifically reacted with the anti-D. burnetii serum (inset), 100×.*

rapidly growing in various directions when examined by inverted phase contrast microscopy at high magnification (1000x). Also the projections booted out from the cytoplasmic membrane and transformed into protrusions of varying thickness and in random directions. Membrane ruffles were also observed as transient cytoplasmic expansions lying between the projections stretching in opposite directions (Fig. 2). Within minutes, a single TOL cell developed into many morphologically different "TOL cell types" distinguished by their locations and shapes of hairy projections (Fig. 3). In addition to their morphological resemblance to HC in HCL, TOL cells demonstrated two other characteristics associated with HC in HCL; TRAP in the cytoplasm and Slg in the cytoplasmic membrane. TOL cells were negative for T-cell markers CD 3, CD 4, and CD 8, and for antigens of HTLV-I, HIV, and EBV. A majority (87 %) of TOL cells displayed hypotetraploid. Their chromosome number varied from 31 to 101 (Table 1).

C. burnetii persisted in TOL cells

When TOL cells were examined by TEM, more than 75% were found to be parasitized by *Coxiella*-like organisms as judged by their size (varying from 0.2 μm to 1 μm in diameter) and by the presence of endospore-like granules in them (Fig. 4). The organisms were also detected in TOL cell's cytoplasmic projections. The organism was specifically identified as *C. burnetii* by IFA (Fig. 3).

Table 1. Characteristics of TOL cell line

Originated from a case with chronic fever, skin infection, pancytopenia
Positive for tartrate resistant acid phosphatase (TRAP)
Positive for surface immunoglobulin (Slg) not for CD 3, CD 4, CD 8
Negative for HIV, HTLV-1 and EBV
Major immunoglobulin secreted into media was isotype: IgG_4
Chromosome range; 31-101, mode number 61-70 (hypotetraploidy)
Persistently infected with *C. burnetii* [strain: Yonsei]*

*Tentative name of *C. burnetii* strain isolated from TOL cells.

HC transformation by *C. burnetii* isolated from TOL

Transformation of PBL into cells with the morphological and phenotypic characteristics of the indicator TOL cell was seen in 6 out of 7 PBL and 2 out of 3 CBL cultures, within 5~45 days of inoculation (average 20 days). All of the new hairy cell lines developed in PBL or CBL cultures by the isolates from TOL cells were positive for Slg and parasitized with *C. burnetii* as determined by IFA. Two third of them were positive for TRAP.

HC transformation by *C. burnetii* passaged in vero and strain nine mile

Within 72 hours of inoculation, infection of Vero cells with *C. burnetii* was confirmed by IFA. Unexpectedly, some of the surviving cells developed long cytoplasmic projections, delicate enough to be visible only by phase contrast microscopy, in 7 days. The organisms passaged in Vero cells for more than two months, when inoculated onto fresh PBL, produced hairy cell transformation (3/3). Cytoplasmic presence of *C. burnetii* was confirmed in the cells by IFA.

With the Nine Mile strain of *C. burnetii*, HC transformations were also produced in PBL cultures (6/6). The morphological and phenotypic characteristics of the Nine Mile strain induced-hairy cells were not distinguished from those of the indicator TOL cell which demonstrated characteristics of HC in HCL.

No HC transformation in lymphoid cell lines by *C. burnetii*

Responses of previously immortalized non-hairy cells to *C. burnetii* super-infection were tested. Established lymphoid cell lines of human T and B-cell (MOLT-4, Daudi and Raji maintained in MEM) were inoculated with *C. burnetii* When established lymphoid cell lines of human T and B-cell cells were observed for more than 45 days after the inoculation, no HC transformation was noticed.

Spontaneous HC development in PBL cultures from coxiellemic cases

Following the presentation of a report on TOL cells at a clinical conference in Seoul, in June, 1991 (Lee 1991), blood samples of patients

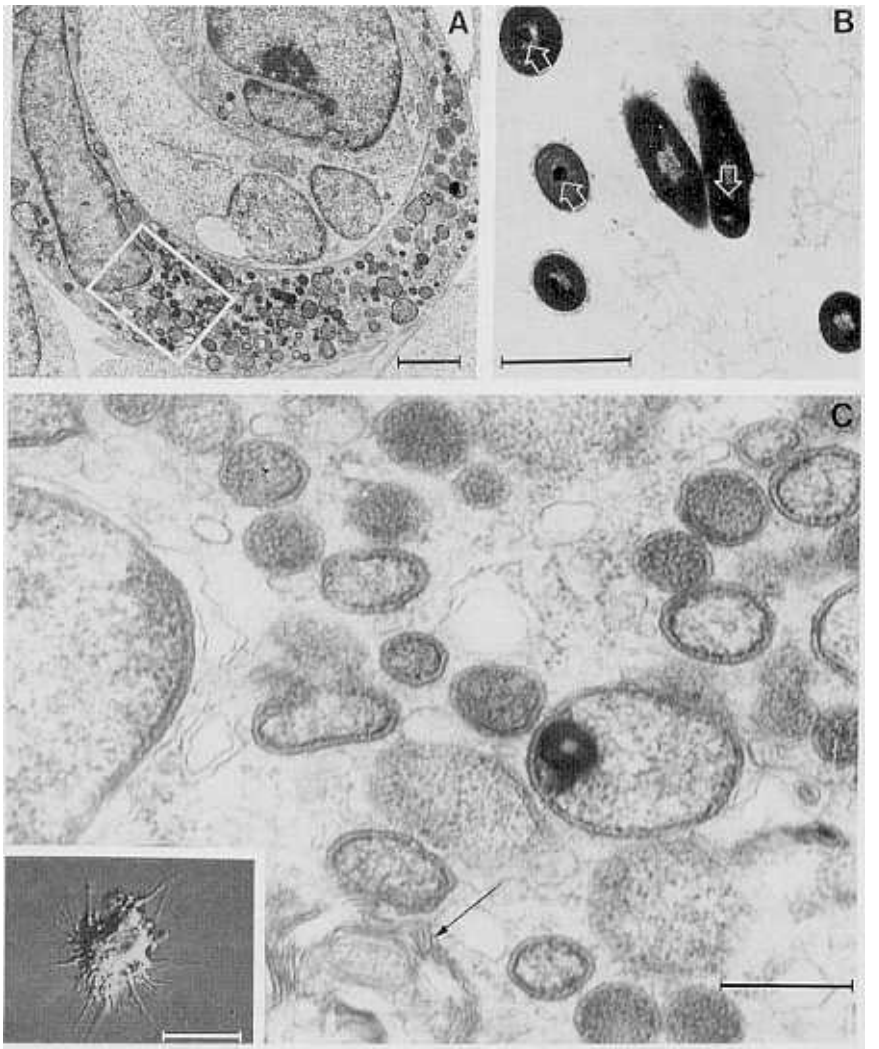

Fig. 4. *Selected photomicrographs of a TOL cell with C. burnetii-like organisms in the cytoplasm and higher magnification of the organisms. Ultrathin sections were fixed in glutaraldehyde and studied by TEM at 3,000 to 30, 000×. (A) Low magnification transmission electron micrograph of cell of which cytoplasm was filled with the organism in various size and shapes.l A part of the cytoplasm marked with square white lines is further magnified. Bar 1 μm. (B) The organisms illustrating the compactness and containing endospore-like granules (arrow). Bar, 1 μm. (C) Numerous double membraned organisms and multiple ribosome lamella complex (arrow). Bar 1 μm. a laser scanning photograph of a TOL hairy cell (inset). Bar 10 μm.*

provisionally diagnosed as various hematologic disorders were sent to my laboratory for a test of *C. burnetii*. Fifty three out of 107 patients were found to be infected with *C. burnetii*. Since the longest time to recognize hairy cell colonies in PBL cultures was 45 days, the 78 cultures (24 positives and 54 negatives for *C. burnetii)* maintained for 45 days or more *in vitro* were analyzed blindly for hairy cell transformation.

Hairy cell transformation was confirmed only in *C. burnetii* positive PBL cultures (12/24) but not in *C. burnetii* negative PBL culture (0/54) as determined by their morphology and by assays

Table 2. Characteristics of HC lines originated from coxiellemic patients with various diagnosis

Patients (n=12)	Sex/ Age	TRAP (n=7)	Slg (n=9)	Isotypes (n=9)	Clinical diagnosis
1	F/3	+	+	IgG_1	HCL
2	F/9	−	−	nt	ALL
3*	F17	+	−	nt	AML
4†	M/50	+	+	IgM	Lymphoma
5	M/41	+	+	IgG_1, IgM	Myelopathy
6	M/47	+	+	IgM	Myelopathy
7	M/54	+	+	IgM	Myelopathy
8	F/54	+	+	IgG_1	FUO
9	F/14	nt	+	IgM	FUO
10	F/5	−	+	IgM	Q fever
11	F/65	nt	+	IgM	HFR
12	M/28	−	−	nt	Contact**

†; Died due to severe pneumonia upon diagnosis.
**; An asymptomatic case whose daughter was a HCL case (YJY).
nt; not tested
ALL, Acute lymphoblastic leukemia; AML, Acute myelocytic leukemia; HCL, hairy cell leukemia; Myelopathy, neurologic symptom due to mass in spinal cord; HFR, Hemorrhagic fever with renal syndrom; FUO, Fever of unkown origin.

for TRAP and Slg in them. Fifty percent (6/12) of the newly established hairy cell lines were positive to both markers, TRAP and Slg, and all were parasitized with *C. burnetii* (Table 2). Cultures of *C. burnetii* negative and/or untransformed PBLs were all degenerated in 45 days *in vitro*.

DISCUSSION

This study demonstrated that *C. burnetii* induces hairy cell transformation in fresh PBL. *Coxiella* induced-HC transformation was produced by all of the three strains of *Coxiella*, the laboratory isolate, Nine Mile strain, and the laboratory isolate passaged in Vero cells. Previously immortalized lymphoid cells inoculated with *C. burnetii* were not transformed. It is well established that EBV specifically immortalizes B cells *in vitro*. However, our results indicate that coexistence of EBV in the inoculum was not required in HC transformation by *C. burnetii* and the HC transformation by *C. burnetii* was not a strain specific event. However, role(s) of latently infected EBV in the fresh B cells before the inoculation, which were not tested in the present study, would have to be cleared. *C. burnetii* caused changes in both morphology and growth behavior of PBL which demonstrated infinite growth *in vitro*. By the Ponten's definition of tumor cells *in vitro* (Ponten, 1971), the HC lines maintained *in vitro* for more than 2 years (2~6 years) are all categorized as cells of "infinite growth transformation". Further study to learn whether HC transformation is a reversible change, especially when the organisms were eliminated, is definitely required.

C. burnetii, the obligatory intracellular parasite, was first identified almost simultaneously in the United States and Australia in 1938-1939 (McDade 1990). The geographic range of the *Rickettsia* is essentially global in wild life and air-borne infection is a major route of human infection. *C. burnetii* is extremely infectious for a non-immune host. One organism is sufficient to initiate infection in guinea pigs (Tigertt *et al.* 1961). In humans, *C. burnetii* has been known to cause Q fever, as an acute febrile illness. It also has been implicated in a number of other diseases; chronic endocarditis, pneumonia, hepatitis, perinatal infection, and pseudotumor of lung (Janigan & Marrie 1983; Lipton *et al.* 1987).

Human disease due to B-cell transformation by *C. burnetii* has not been documented. However, there have been numberous studies on the transformation of *C. burnetii* infected B-cells in morphology and proliferative behaviors. An extensive proliferation of B-cells in animals infected with *C. burnetii* was reported by Khavkin (1990). *C. burnetii* can colonize both phagocytic and non-phagocytic cells *in vivo* (Handly *et al.* 1967). B-cell tropism of *C. burnetii*, especially for the persistent infection, was reported in animal experiments (Khavkin & Tabibzadeh, 1988). *C. burnetii* in vacuoles of B-cells were reported not to harm the essential functions and multiplication of these cells. Association of the proliferative behavior of *C. burnetii* infected B cells and pathogenesis of the splenomegaly in coxiellosis was reported (Khavkin 1990). Since *C. burnetii* produces lipopolysaccharides (LPSs; Amano *et al.* 1987; Williams & Cantrell 1982). Continuous exposure of B cells to LPSs, a potent B cell mitogen, may be responsible for the proliferation of B cells.

Extrusion of the cytoplasmic vacuoles bearing *C. burnetii* by periodic contractions was reported (Khavkin & Amosenkova, 1981). And dendritic, veiled cells named "limbocytes" have been reported in studies of some *Coxiella*-bearing stromal cells in lymphnodes and spleens (Pough & MacPherson 1985; Hume *et al.* 1983). The morphological changes may not be specific to B cells since we noticed similar cytoplasmic changes in some of the Vero cells infected with *C. burnetii*. These cytoplasmic changes in *C burnetii* infected-Vero cells have been described previously by Burton *et al.* who designated the cells as "podocytes" in 1978 (Burton *et al.* 1978). Mechanisms involved in the cytoplamic projections of *C. burnetii* infected cells are not known. Cytoskeletal changes due to LPSs produced by *C. burnetii* may be responsible for those changes since development of filamentous arrays in human B-cells treated with purified LPSs have been observed (Albrecht *et al.* 1990).

An intriguing fact is that electron microphotographies of cytoplasmic granules reported in HCL are morphologically indistinguishable from those of *C. burnetii*. Although *C. burnetii* have never been mentioned in HCL in those reports, unidentified cytoplasmic granules had been frequently reported. In 1972, the cytoplasmic granules in HC of HCL were firstly reported by Katayama *et al.* who described light electron microscopic characteristics of the inclusion bodies in HC of HCL (Katayama *et al.* 1972). Since then, similar observations in HCL had been accumulated as "small and large granules which were electron dense and membrane bound" in more than 50% HC of HCL variants (Catovsky *et al.* 1984) and "abundant mitochondria" and a lamellar complex in HC (Faguet *et al.* 1988). The prognostic significance of cytoplasmic inclusions in HC were reported by Bartl *et al.* who demonstrated an inverse relationship between the amount of cytoplasmic granules in HC and the survival of the HCL patients following treatments (Bartl *et al.* 1983).

At present, it is not known whether the HC induced by *C. burnetii* could be a pathogenic entity of HCL. However, many epidemiological and clinical characteristics of HCL coincide with those of coxiellosis. Characteristics of HCL such as male preponderance, hepatosplenomegaly, pancytopenia, presence of lymphocytes with membrane bound cytoplasmic granules of unknown nature were also reported in coxiellosis. Favorable response of patients with HCL to interferon (Dalal & Fitzpatrick, 1991) are all reported in coxiellosis too.

We experienced a patient with HCL (included in Table 2) who had completely recovered from the disease by use of antibiotics recommended for coxiellosis (in preparation). Additional supportive data are now accumulating in clinical studies. In this regard, a case of HCL associated with Q fever has been recently reported (Vuille & Delafontaine, 1989). Thus, "HCL-like" disease due to *C. burnetii* infection may be possible especially in regions where *C. burnetii* is endemic. Fortunately, *C. burnetii* is susceptible to antibiotics and vaccines are available for the exposures.

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