tion of the frequency of confined chromosomal mosaicism, its association with IUGR, and its possible association with preterm unexplained intrauterine death is needed.

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Hepatitis B Virus Infection in Cultured Human Lymphoblastoid Cells

Abstract. *Since it has been postulated that liver hepatocytes may become infected by hepatitis B virus (HBV)* in vivo *through direct contact with infected macrophages, the possibility that a circulating cell of hematopoietic origin might be susceptible to infection with HBV was investigated. Cells positive for HBV surface antigen were identified in aspirates of bone marrow cells from people infected with HBV. These cells were used to prepare a lymphoblastoid suspension culture that contains HBV-infected cells.*

Research progress with hepatitis B virus (HBV) has been hampered by the lack of a cell culture system for growing the agent. It has been postulated that liver hepatocytes may become infected by HBV in vivo through direct contact with infected macrophages (*1*). This raises the possibility that liver macrophages or a circulating cell of hematopoietic origin might be susceptible to infection with HBV. To address this issue we examined aspirates of bone marrow cells from people who were infected with HBV. After identifying HBV surface antigen (HBsAg)-positive cells in such biopsies we established a lymphoblastoid suspension culture that contains HBV-infected cells.

At present, the only available cultures that remain persistently infected with HBV are liver tumor cell lines derived from HBV-infected people (*1–5*) and cells transfected with HBV DNA (*6*). Neither type of culture appears to make whole virus or to release HBV into the culture supernatant. Most produce only HBsAg, although some transfected cells appear to make virion core antigen (HBcAg), at least for limited periods.

Bone marrow aspirates from four HBsAg-positive and five HBsAg-negative patients with various hematologic diseases were tested under code for HBsAg. Smears of acetone-fixed cells were tested by direct immunofluorescence with a 1:20 dilution of rabbit antiserum to HBsAg conjugated to fluorescein isothiocyanate (FITC) (Behring). Samples from the four HBsAg-positive patients revealed that 1 to 2 percent of the cells were positive for cytoplasmic fluorescence, while the other five were negative. A subsequent bone marrow aspirate was available from one of the four patients, and this was used to initiate a cell culture.

The donor was a 55-year-old anemic white male (RAC) who was hospitalized for acute hepatitis. The patient had been on dialysis for 1 year and had received multiple transfusions. He was serologically positive for HBsAg, HBV e antigen, and had antibodies to HBc. Three months after diagnosis the signs and symptoms of hepatitis were partially resolved and a bone marrow aspirate was checked for anemia. At this time a portion of bone marrow containing approximately 10^7 mononuclear cells was seeded in RPMI 1640 medium with 25 percent heat inactivated fetal calf serum and, per milliliter, 10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of Fungizone (Gibco). This culture, designated RAC/BM and incubated at 37°C in 5 percent CO_2, has been maintained in suspension form for 10 months. It is subcultivated at 3- to 4-day intervals by disruption of the cell clusters and reseeding at a density of 5×10^5 cells per milliliter.

By examination with the light microscope the cells are round but pleomorphic, and some have small cytoplasmic projections. When examined with the May Grünwald Giemsa stain, most resemble lymphoblasts (Fig. 1). Karyotypic analysis indicated that the RAC/BM cells contained 46 chromosomes. Smears of RAC/BM were also stained with the periodic acid–Schiff (PAS) reagent. Forty to 50 percent of the cells were PAS-positive, whereas all were negative for chloroacetate esterase peroxidase and

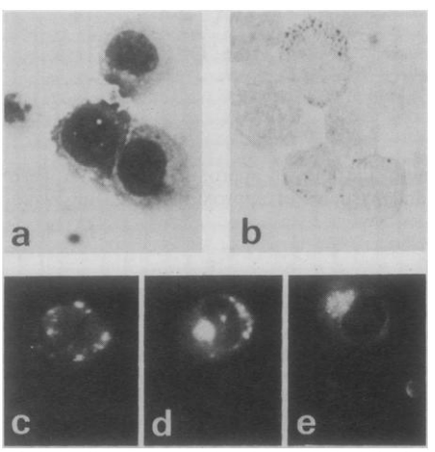

Fig. 1. Characteristics of RAC/BM cells at passage 31. (a and b) Smears of cells stained with May Grünwald Giemsa and PAS. (c) Living cells (10^6) were washed three times with phosphate-buffered saline (PBS), incubated with 50 μl of rabbit antibody to HBsAg conjugated with fluorescein (Behring) for 1 hour at 4°C, and washed again three times before examination. Whereas 20 percent of the RAC/BM cells were positive with either this technique or an indirect technique in which a human antiserum to HBsAg was used together with a conjugated rabbit antibody to human immunoglobulin G (Behring), less than 1 percent of the cells of a negative control line (CCRF-SB) (*10*) were positive and 10 to 15 percent of the PLC/PRF/5 cells were positive. (d and e) In air-dried smears of RAC/BM fixed in acetone at −20°C for 10 minutes, 15 percent of the cells were positive for cytoplasmic fluorescence when tested with human antiserum to HBsAg (d) and 12 percent were positive with human antiserum to HBcAg (e). The F(Ab)′ fragment of goat immunoglobulin directed to human immunoglobulin G labeled with fluorescein (Cappel) was used and each incubation step was for 40 minutes at 37°C. Less than 1 percent of the control cells were positive with either antiserum.

N-butyl acid esterase (Fig. 1). When taken in combination, these results suggest that the culture is composed primarily of lymphoblasts.

The RAC/BM cells were then tested for the Epstein-Barr virus nuclear antigen (EBNA) by means of fixed-cell anticomplement immunofluorescence (*7*). When 200 cells of each culture were counted, 53 percent of the RAC/BM were positive; 83 percent of the positive control cells (B95-8) (*8*) were positive; and less than 1 percent of the negative control cells (Ramos) (*8*) were positive.

From 17 to 20 percent of RAC/BM cells were positive for HBsAg by direct and indirect immunofluorescence at passages 12, 20, and 31 when tested by the living-cell membrane technique. HBsAg and HBcAg were also detected by direct and indirect immunofluorescence in the cytoplasm of RAC/BM cells in acetone-fixed smears. Fifteen percent of the cells were positive for HBsAg and 12 percent were positive for HBcAg. No nuclear fluorescence was observed (Fig. 1).

HBsAg-positive cells were concentrated by means of the fluorescence-activated cell sorter (FACS II) and then examined for HBcAg in the cytoplasm. Whereas 36 percent of the cells from the RAC/BM culture that was enriched for HBsAg positivity were also positive for HBcAg, only 9 percent of the reciprocal population was positive for HBcAg. The population that was enriched for HBcAg was also drastically reduced in the number of EBNA-positive cells. To determine if HBsAg was present in the RAC/BM supernatants, we collected 200 ml and after concentrating it passed it through a discontinuous cesium chloride gradient. The gradient fractions were tested for HBsAg by radioimmunoassay (RIA; Abbott) and examined by electron microscopy after negative staining. Fractions 1.18 to 1.25 g/cm^3 were positive for HBsAg, and isolated particles of the same size and shape that were described earlier in plasma as Dane particles (*2*) were seen in the 1.23 g/cm^3 fraction (Fig. 2). Examination with the electron microscope showed that the Dane-like particles were aggregated after incubation with antibodies to HBsAg (Fig. 3).

As another method of evaluating the presence of HBsAg in RAC/BM cells, we injected rabbits with lethally irradiated cells. Before every injection of cells, serum samples were tested by RIA for antibodies to HBsAg. As indicated in Table 1, approximately the same amounts of antibodies to HBsAg were seen when either RAC/BM or PLC/PRF/5 cells were injected. PLC/PRF/5 is a HBsAg-producer positive reference hepatoma line (*3*). When taken in combination, these results indicate that some patients that are infected with HBV may harbor the virus in selected populations of bone marrow cells. We cultivated cells from bone marrow of an infected patient and showed, by means of direct and indirect immunofluorescence, radioimmunoassay, and electron microscopy, that antigens specific for HBV infection (HBsAg and HBcAg) are present in the RAC/BM cells. Structures that morphologically resemble Dane particles and HBsAg aggregates were also found in the culture supernatant. Experiments suggest that HBV-specific DNA sequences may be present in RAC/BM (*9*).

It appears likely that the RAC/BM culture contains a mixed population of suspended cells. The predominant population in the culture is an EBNA-positive B cell; that population does not appear to

Table 1. Detection of radioimmunoprecipitating antibodies to HBsAg in rabbits inoculated with HBsAg or cultured cells. Rabbits 2 to 5 were injected on days 1, 9, 17, and 25. Rabbit 2 received HBV Pasteur Institute vaccine (lot 04A). Rabbits 3 to 5 received 3 × 10^7 irradiated cells in Freund's complete adjuvant at the first injection and the same number of cells without adjuvant at each subsequent injection. Rabbit 2 developed an antibody titer of 1:10,000 by day 32 whereas rabbits 3 and 4 developed titers of only 1:4 by day 32.

Rabbit No.	Inoculum	Days after first inoculation				
		0	8	16	24	32
1	Uninoculated	–	–	–	–	–
2	HBsAg vaccine	–	–	+	+	+
3	RAC/BM cells	–	–	–	+	+
4	PLC/PRF/5 cells	–	–	–	+	+
5	Hep G2 cells*	–	–	–	–	–

*Described in (*4*).

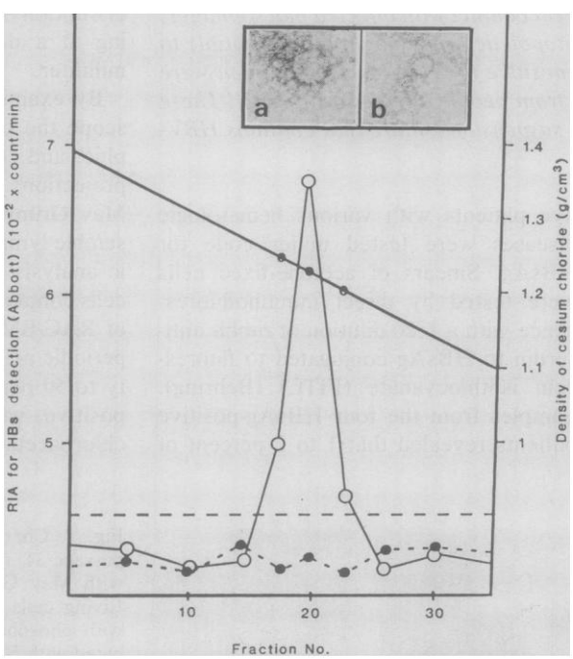

Fig. 2. Cesium chloride gradient analysis of 1 ml of 200-fold concentrated supernatant from RAC/BM cells (○) and control peritoneal fibroblasts (RAC/F) (●) from the same patient. The RAC/BM supernatant was used at passages 25 to 30. The culture fluid, collected at the time of each subcultivation, was initially clarified by centrifugation at 1500*g* for 30 minutes and then at 40,000*g* for 30 minutes. The resulting supernatant was then centrifuged at 100,000*g* for 18 hours. The resulting pellet was dissolved in tris buffer at *p*H 7.5 with 0.005*M* EDTA and layered on a discontinuous cesium chloride gradient of 12 percent, 22 percent, and 32 percent and spun for 20 hours at 100,000*g*. Fractions of 1 ml were then dialyzed overnight against PBS and tested by RIA for HBsAg. Those that were positive were also examined with the electron microscope after negative staining. Particles of approximately 45 nm were observed in the 1.25 g/ml density fraction (a), and particles of approximately 23 nm were observed in the 1.18 g/ml density fraction (b).

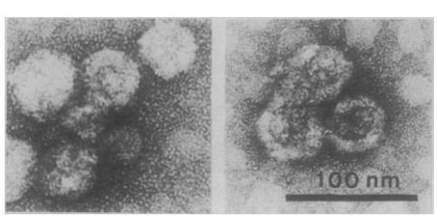

Fig. 3. Electron microscopy of supernatant from passages 25 to 30 of RAC/BM. The supernatant (200 ml) was concentrated and gradient-separated as in Fig. 2. The fraction at 1.25 g/ml (0.1 ml) was mixed with 0.1 ml of a 1 in 100 dilution of rabbit antiserum to HBs (rabbit No. 2 in Table 1), incubated at 37°C for 1 hour and then at 4°C for 16 hours. This mixture was then diluted in 10 ml of PBS and centrifuged at 48,000*g* for 1 hour. The resulting pellet was resuspended in 0.1 ml of PBS and dropped on a carbon-coated grid, and 1 percent uranyl acetate was added for negative staining. The preparation was then examined with a Zeiss 10 electron microscope.

contain many HBsAg-positive cells. The population enriched for HBsAg with the cell sorter was markedly reduced for the proportion of EBNA positive cells. Attempts to clone the HBV-infected cells are underway. However, it is possible that the RAC/BM cells requires the presence of the population infected with Epstein-Barr virus or factors produced by the latter cells for continuous growth in culture.

The proportion of cells infected in the mixed RAC/BM culture fluctuates from 10 to 20 percent, and the amount of HBsAg produced appears to be low. Nevertheless, positive cells were still present after more than 10 months in culture, and the proportion remained higher than the proportion of cells that were positive in the fresh bone marrow population.

The presence of HBcAg in some of the cells and the detection of virus-like particles at the density expected for Dane particles suggests that the RAC/BM culture might produce infectious HBV. Again, however, based on the amount of HBsAg detected, the numbers of virus-like particles produced is probably quite low.

The only pathological lesions regularly associated with HBV infections are those found in the liver. Perhaps because of this many virologists have assumed that hepatocytes and liver macrophages (Kupffer cells) are the only major cellular target for infection in vivo. Several cultures have been established from patients with primary liver cancer. At least two, PLC/PRF/5 and Hep 3B, appear to contain HBV genome and to make HBsAg in large quantities (*3*, *4*). Both are composed of adherent epithelial cells which presumably represent malignant hepatocytes, but neither culture appears to make whole virus. Our studies suggest that bone marrow should also be considered as a possible site for HBV infection in vivo and that various cell populations of hematopoietic origin should be evaluated for susceptibility to HBV replication both in vitro and in vivo.

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Low Nitrogen to Phosphorus Ratios Favor Dominance by Blue-Green Algae in Lake Phytoplankton

Abstract. *An analysis of growing season data from 17 lakes throughout the world suggests that the relative proportion of blue-green algae (Cyanophyta) in the epilimnetic phytoplankton is dependent on the epilimnetic ratio of total nitrogen to total phosphorus. Blue-green algae tended to be rare when this ratio exceeded 29 to 1 by weight, suggesting that modification of this ratio by control of nutrient additions may provide a means by which lake water quality can be managed.*

The ability to predict and manage algal biomass and transparency in lakes has been greatly improved by the development of empirical models of eutrophication due to phosphorus loading (*1*). However, whether specific lake restoration measures currently in use will significantly reduce the proportion of nuisance blue-green algae (Cyanophyta) in the epilimnetic phytoplankton cannot yet be predicted with confidence.

Numerous hypotheses have been proposed to explain the success of blue-green algae in eutrophic lakes (*2*). Although the importance of nitrogen to phosphorus (N:P) ratios in determining algal blooms has been discussed since the work of Pearsall (*3*) and Hutchinson (*4*), there have been comparatively few direct studies of the relation between N:P ratios and the presence of blue-green algae (*5*, *6*). I report a dramatic tendency for blue-green algal blooms to occur when epilimnetic N:P ratios fall below about 29:1 by weight, and for blue-green algae to be rare when the N:P ratio exceeds this value.

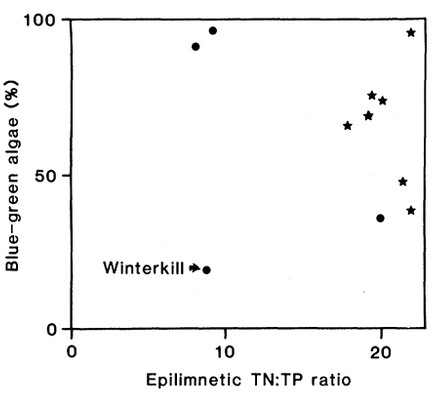

Fig. 1. Relation between the growing season mean proportion of blue-green algae and epilimnetic total nitrogen (*TN*) to total phosphorus (*TP*) ratios in Lake Trummen, Sweden. All blue-greens (unicellular, colonial, heterocystous, and nonheterocystous filamentous species) were included in the calculation of their proportion in the phytoplankton (*11*). Symbols represent data from one growing season for 11 years of measurements (*13*); circles indicate years before dredging, and stars, years after the lake was dredged.

The nutrient physiology of the Cyanophyta differs from that of other algae in that many blue-green species are capable of nitrogen fixation. This ability allows nitrogen-fixing species to maintain high growth rates in habitats deficient in inorganic nitrogen, and they should thus be superior nutrient competitors under conditions of nitrogen limitation. In addition, blue-green species that apparently do not fix nitrogen, such as *Microcystis aeruginosa*, may be as abundant as nitrogen-fixing forms during times of nitrate deficiency (*7*). In contrast, Tilman *et al.* (*8*) suggested that blue-green algae (both those that fix nitrogen and those that do not) are generally inferior to diatoms as phosphorus competitors, indicating that blue-green algae should typically be dominant in lakes with low N:P ratios (in which most phytoplankton species would be nitrogen-limited) and rare in lakes with high N:P ratios. Flett *et al.* (*9*) found that nitrogen-fixing blue-green algae were typically associated with lakes