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In Vitro Inhibition of Hemopoietic Cell Line Growth by Hepatitis B Virus

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The effects of hepatitis B virus (HBV) on established human cell lines of various tissue origins were evaluated by clonal or colorimetric assays in methylcellulose culture. HBV exposure inhibited the growth of six hemopoietic cell lines, while similar incubation did not affect the growth of seven nonhemopoietic carcinoma cell lines of breast, colon, liver, and stomach origin. The inhibition of hemopoietic cell line colony formation was dependent on the presence of intact viral (Dane) particles and the ratio of exposure of virions to cells and was reversible with antibodies to pre-S1, pre-S2, and S envelope protein epitopes. Purified HBV DNA, surface antigen pre-S antigens, and core antigen did not inhibit cell line growth. These results further demonstrate the tropism of HBV for cells of hemopoietic origin, confirming our previous findings on the effects of HBV on the growth of normal bone marrow progenitor cells in vitro. Established human tissue culture cell lines may be used to study the interactions of hemopoietic cells with HBV.

Hepatitis B virus (HBV) is not exclusively hepatotropic. HBV DNA has been detected in pancreas, kidney, skin (4), bone marrow (5, 9), and peripheral blood mononuclear cells (7, 8). Romet-Lemonne et al. (9) and Elfassi et al. (5) have shown that viral antigens and HBV DNA are present in a small percentage of bone marrow cells from infected patients. We have found that the in vitro growth of human bone marrow progenitor cells is suppressed after exposure to HBV (15). This inhibition is dependent on the ratio of the number of virions per mononuclear cell and can be blocked by antibody to HBV surface antigen (HBsAg) (14). Since cell lines of hemopoietic origin are more readily available than normal bone marrow cells, it was of interest to examine the effects of HBV on the growth of cell lines as a potential model for HBV infection.

MATERIALS AND METHODS

Virus. Virus-containing sera were obtained from patients with chronic HBV infections. The sera lacked serologic evidence of human immunodeficiency virus infection. Sera were filtered through 0.22-mm-pore-size filters and stored at −20°C. Virion (Dane particle) preparations (14) and plasma and recombinant-derived small HBsAgs were gifts of William Miller, Merck, Sharp, Dohme (West Point, Pa.). HBV was also produced by growing, in RPMI 1640 with 10% fetal bovine serum (FBS), a hepatoblastoma cell line, HepG24, that was transformed with HBV DNA, obtained from G. Acs, Mt. Sinai School of Medicine, New York, N.Y. The supernatant typically had between 10^6 and 10^7 virions per ml. A crude purification was made by pelleting the virus for 2 h in RPMI 1640 at 45,000 rpm in a Beckman 50.2Ti rotor at 10°C. Recombinant HBV core antigen (HBcAg) was a gift from Biogen, Cambridge, Mass. HBV DNA from HBV-containing sera that inhibited human hemopoietic stem cell colony formation was purified by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The concentration of viral DNA was determined by DNA spot hybridization assays with cloned HBV DNA as the standard (13). The concentration of virus was estimated by assuming that all HBV DNA was encapsulated in a virus. This was used to calculate the ratio of exposure (ROE) of the number of virions to cells in all experiments.

Cell lines. Six human cell lines of hemopoietic origin and three of nonhemopoietic origin were obtained from the American Type Culture Collection. K562, HL60, and U937 are myeloid leukemic lines. CEM and Jurkat cells are of T-cell origin, and JY is of B-cell lineage. These lines were grown in suspension culture in Iscove modified Dulbecco medium (IMDM) supplemented with 10% heat-inactivated (56°C for 30 min) FBS. T47D is derived from a human breast carcinoma, CCL-247 is from a gastric carcinoma, and B5637 is from a bladder carcinoma. These nonhemopoietic cell lines were grown as monolayers in IMDM with 10% heat-inactivated FBS. Like the hemopoietic lines, these three nonhemopoietic cell lines are capable of forming colonies in semisolid agar or methylcellulose. The liver-related cell line PLC/PRF/5 was obtained from the American Type Culture Collection (Bethesda, Md.); HepG2 was from B. Knowles (Wistar Institute, Philadelphia, Pa.); and two stable HBV transformants of HepG2 that produce virus, HepG2T14, from M. Essex (Harvard School of Public Health, Boston, Mass.), and HepG24, from G. Acs (Mount Sinai School of Medicine, New York, N.Y.), were obtained.

Colony assay. Leukemic cell lines (300 cells per culture) were incubated at 37°C and 5% CO_2 overnight with HBV DNA-containing sera, virions, or purified HBV-related antigens or DNA. Cells exposed to normal human AB sera served as controls. The cells were then plated in triplicate in IMDM containing 0.9% methylcelluose (Fisher Scientific), 20% FBS, and 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). Colonies of greater than 30 cells were scored after 10 days of culture. The number of colonies

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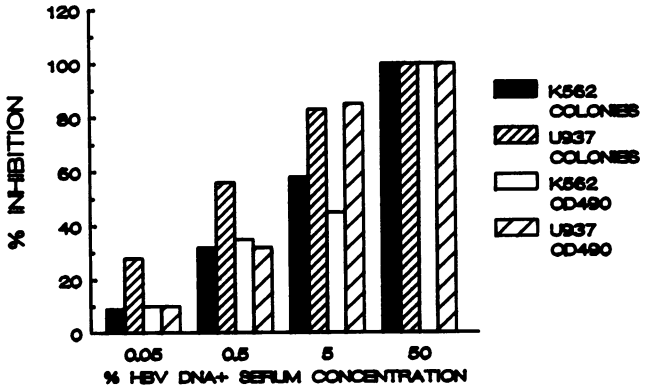

FIG. 1. HBV-containing serum inhibits colony formation and cell growth as measured by MTT assay of K562 (open and solid bars) and U937 (hatched bars) cells. The number of colonies in the control was 57 ± 2 for K562 and 34 ± 2 for U937.

formed in the experimental group was compared with the number formed after incubation with control sera. The data are expressed as percent inhibition.

Cell growth was alternatively studied by a modified assay (2). This assay measures labile cellular dehydrogenases that are only found in viable cells. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide (MTT; Sigma) was added to each culture and incubated for 4 h at 37°C and 5% CO_2. The cultures were harvested, and the formazan particles were pelleted and solubilized in 100% dimethyl sulfoxide (Sigma). A portion of the solution was transferred to a 96-well plate, and the OD_{490} was measured.

Antibody neutralization studies. Before the cells were exposed to virus, HBV preparations (sera or purified virions) were incubated for 4 h at 37°C with polyclonal antisera, supernatants from hybridoma cultures, or diluted murine ascites containing monoclonal antibodies. The percent neutralization was calculated by the following formula: $[1-(N_{Ab}/N_v)] \times 100$, where N_{Ab} is the number of colonies or percent change in MTT value obtained by incubating HBV with antibody (Ab) prior to exposure of the cells, and N_v is the number of colonies or percent change in MTT value obtained by exposing the cells to HBV alone. The 5D3 murine monoclonal antibody is a monomeric immunoglobulin M (IgM) with high avidity for HBsAg (K_m, $>10^{11}$), obtained from J. Wands, Massachusetts General Hospital, Boston, Mass. (12). The anti-measles virus murine monoclonal antibody supernatant was the gift of C. Desgrange, Institut National de la Santé et de la Recherche Médicale, U271, Lyon, France.

RESULTS

The results of the colony-forming assay and the MTT assay correlated well with each other. Serum devoid of HBV did not affect the growth of any cell line as measured by either assay. Incubation of hemopoietic cell lines K562 and U937 with serial dilutions of HBV DNA sera resulted in a dose-dependent inhibition of their clonal growth in methylcellulose as measured by either assay (Fig. 1). The number of cells in the colonies that formed in the presence of HBV was no different than that found in cultures of cells not exposed to virus. After HBV exposure, many cells were found in suspension, not associated with colonies. Most of these cells were viable after 5 days in culture in that they

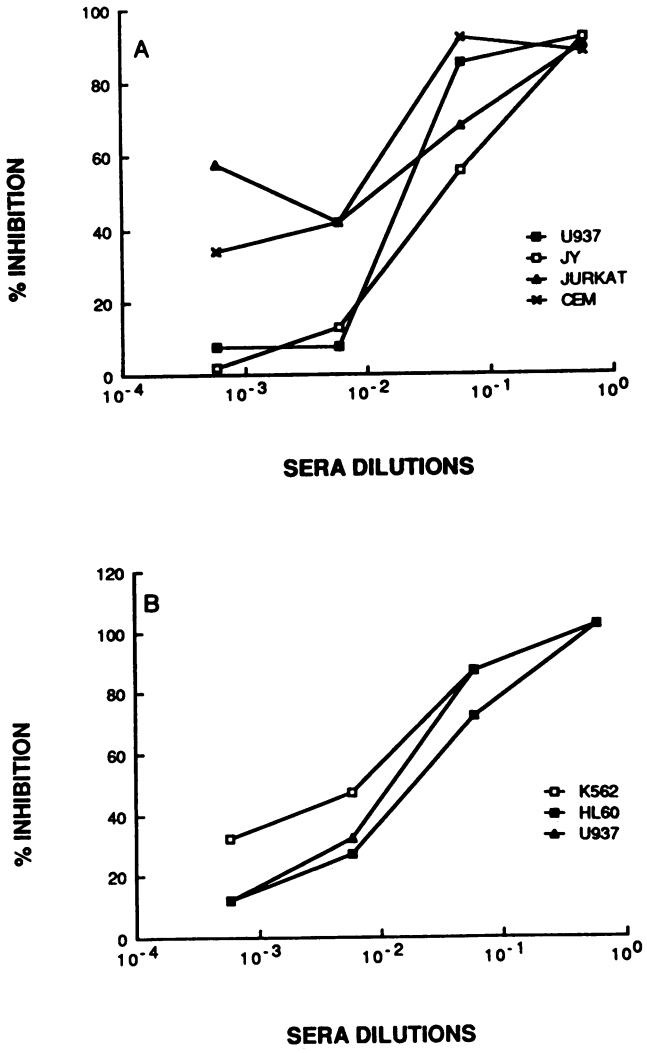

FIG. 2. Effect of HBV-positive sera on hemopoietic cell lines. (A) Effects on cell growth determined by MTT assay. The OD_{490} of the controls was 0.290 ± 0.006 for U937, 0.392 ± 0.006 for JY, 0.248 ± 0.0 for Jurkat, and 0.215 ± 0.004 for CEM. (B) Effects on colony formation. The control number of colonies for K562 was 57 ± 2 for K562, 34 ± 2 for U937, and 122 ± 3 for HL60.

excluded trypan blue stain. HBV-containing sera inhibited the growth of all six human hemopoietic cell lines in a dose-dependent manner (Fig. 2), while the same sera did not inhibit the growth of three nonhemopoietic cell lines of breast, colon, and stomach origin (Fig. 3). The growth of liver-derived cell lines PLC/PRF/5, HepG2, HepG2T14, and HepG24 was also unaffected by HBV (data not shown). The last two lines were actively making HBV. Hemopoietic cell lines derived from T cells (CEM and Jurkat) appeared to be more sensitive to HBV than lines of myeloid (HL60, U937) or B-cell (JY) origin. At a 1:1,000 dilution of sera, inhibition of the T-cell lines occurred, whereas little or no suppression was observed in U937, HL60, and JY cells (Fig. 2).

The degree of K562 and HL60 inhibition was proportional to the ROE of virus to cells, as determined by the number of HBV DNA molecules incubated per cell (Fig. 4). Complete inhibition of K562 and HL60 growth was observed at an ROE of 3×10^3 virions per cell. Significant suppression of

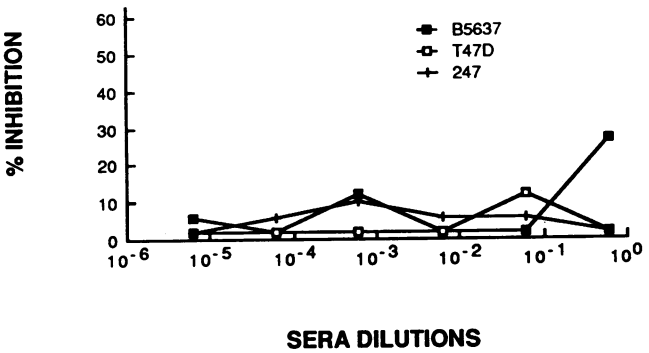

FIG. 3. Effect of HBV-positive serum on the growth of three nonhemopoietic cell lines by the MTT assay. This is the same serum used for the experiments shown in Fig. 1 and 2. The OD_{490} of the controls was 0.220 ± 0.020 for T47D, 0.233 ± 0.010 for B5637, and 0.540 ± 0.010 for C247.

K562 was still observed at 3 virions per cell. Experiments with purified virions were performed to eliminate the possibility that factors in HBV-containing sera other than virus were responsible for the suppressive effect on hematopoietic cell line growth. An ROE of 3×10^4 virions per cell inhibited K562 and HL60 cells by 45 and 30%, respectively (Fig. 5A). In all experiments, more than 1 virus per cell was required to observe cell growth inhibition. Virus purified from the supernatants of HepG24 cells also inhibited colony formation. While both HBV-containing sera and purified virions inhibited hematologic cell line growth, heat-inactivated virions, purified plasma-derived small HBsAg and yeast-derived recombinant small HBsAg, recombinant HBcAg, pre-S1 and pre-S2 synthetic polypeptides, and purified viral DNA derived from sera did not suppress K562 or HL60 colony formation even at concentrations consistent with a very high ROE (Fig. 5C).

Antibodies to surface epitopes on virions neutralized the HBV-mediated inhibition of cell line growth (Fig. 5). At an ROE of 3×10^4 virions per cell, 1:10 and 1:100 dilutions of murine ascites containing 5D3, an anti-HBsAg monoclonal antibody, neutralized the inhibitory effects of HBV on K562 and HL60 growth (Fig. 5B). The neutralizing effect of the antibody was no longer evident at dilutions greater than 1:1,000, where suppression by HBV was again observed. At a dilution of 1:100, 5D3-containing murine ascites completely blocked the virion preparation-mediated dose–response suppression of K562 and HL60 cells (Fig. 5A). Other monoclonal antibodies and polyclonal sera that bind pre-S2 and S determinants also neutralized the HBV-mediated inhibitory effects. A polyclonal serum that only binds the pre-S1 epitope and not the S and pre-S2 epitopes also neutralized the HBV-mediated inhibition of K562 colony formation (data not shown). Furthermore, murine monoclonal antibodies to human immunoglobulins, duck hepatitis virus, and measles virus did not neutralize HBV-mediated inhibition of colony formation. An antibody to woodchuck hepatitis virus that cross-reacted with HBsAg neutralized the inhibition. The neutralization of 5D3 could be abrogated by incubating this antibody with HBsAg and pre-S antigens purified from serum (Fig. 5D). Thus, epitopes to the envelope proteins of HBV are required for the inhibition of K562 colony formation, but isolated envelope proteins are not sufficient to inhibit colony formation. This implies that intact virus is needed for this effect to be observed.

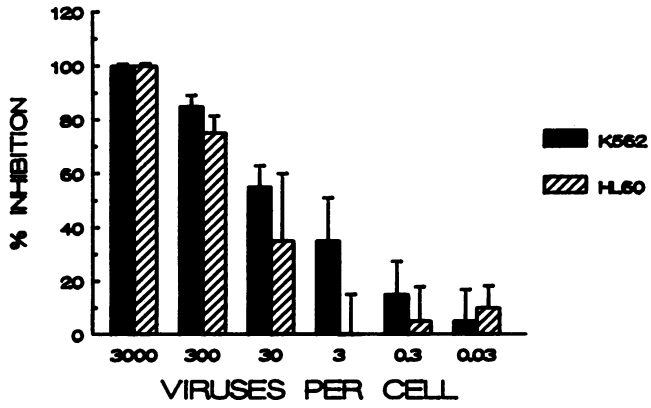

FIG. 4. HBV-mediated inhibition of K562 and HL60 growth with a serum sample from a patient with chronic active hepatitis is dependent on the ROE (the number of virions per cell). The number of virions per cell was estimated from the concentration of HBV DNA. The number of colonies in the control was 81 ± 7.6 for K562 and 69 ± 6 for HL60.

DISCUSSION

HBV infections are commonly accompanied by transient bone marrow depression and rarely by severe bone marrow failure. Infection of the blood-forming cells of the bone marrow by HBV may be involved in these two clinical effects. In this regard, we have reported that HBV inhibited human bone marrow progenitor growth in vitro (14, 15). Incubation of bone marrow cells with HBV resulted in the inhibition of erythroid, granulocyte/macrophage, lymphocyte, and pluripotential progenitor cells. While the mechanism for this effect is not understood, we have observed that viral DNA sequences are present in both human bone marrow mononuclear cells and hemopoietic cell lines after exposure to virus-containing sera and virion preparations. When a virus sample does not inhibit colony formation, no viral DNA is observed in these cells (Bouffard, unpublished data).

In the present study, the effects of HBV on human cell lines of hemopoietic origin are consistent with our previous findings with human bone marrow. The lack of an effect by purified HBsAg, HBcAg, and pre-S antigens as well as purified viral DNA suggests that an intact virus is required to inhibit cell growth. The suppression of hemopoietic cell growth appears to be tissue specific, since no inhibition was observed with the nonhemopoietic cell lines studied. While inhibition of all cell lines required an ROE exceeding 1 virus per cell, different lines were variably sensitive to HBV exposure. The differential sensitivities of these cell lines to HBV may be a function of their relative state of differentiation and the cell type they represent. The most sensitive cells were CEM and Jurkat, lines of T-cell origin, and K562, a cell with pluripotential characteristics. The more mature myeloid lines, U937 and HL60, and the B-cell-derived line, JY, were less sensitive to HBV.

Both sera containing virus and purified virions inhibited colony formation. Furthermore, colony formation was also inhibited by virus derived from HepG24 cells (Fig. 5D), which has been demonstrated to be infectious for chimpanzees (1). As with the human progenitor cell assays, a lower ROE with HBV-containing sera was required to inhibit cell growth than with purified virus in the Dane particle preparations (14). This difference may reflect inactivation of

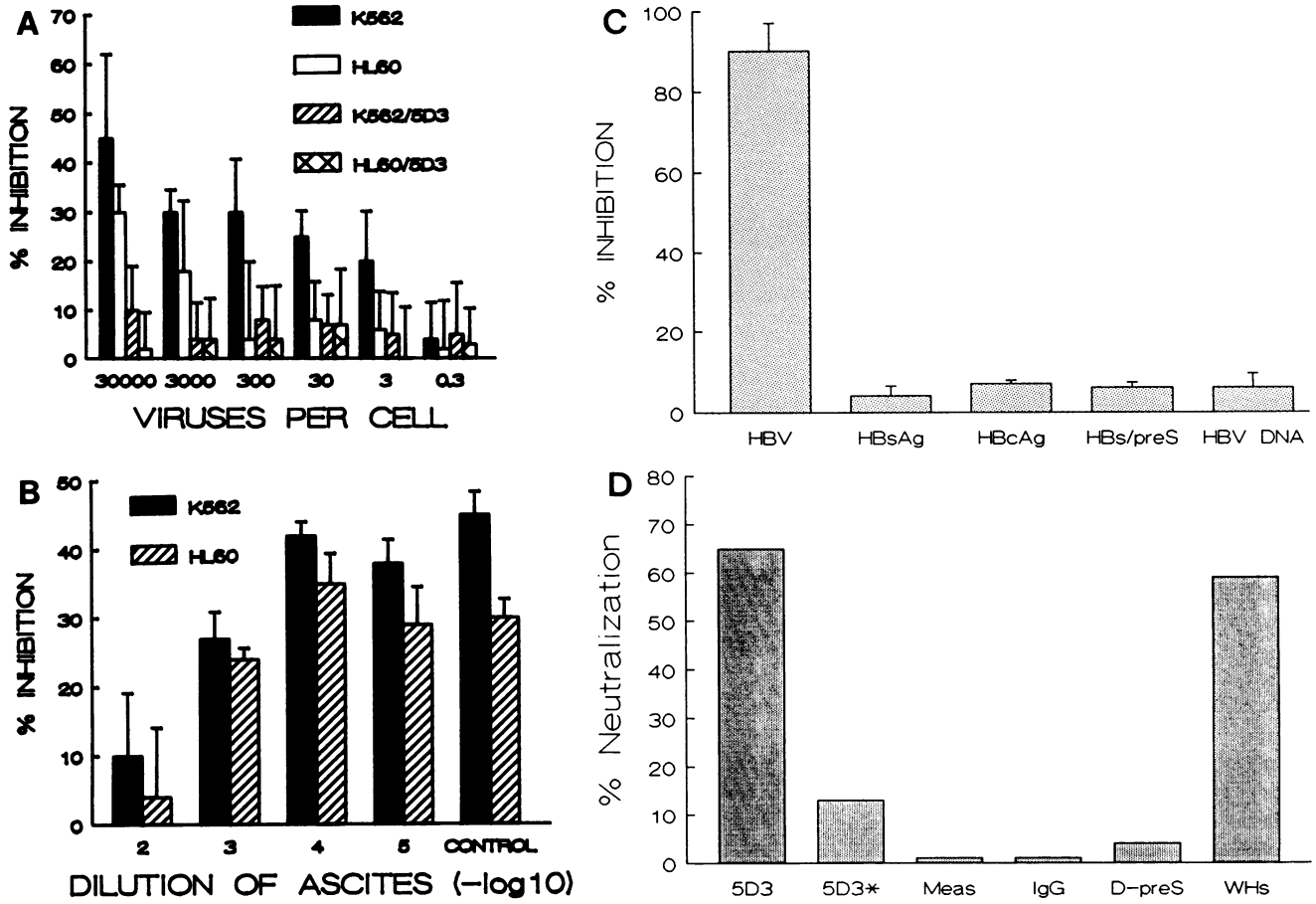

FIG. 5. Effect of anti-HBsAg antibodies on Dane particle-mediated inhibition of colony growth. (A) K562 and HL60 cells were grown with virions or with virions preincubated with 5D3 antibodies for 4 h at 37°C. Ascites containing 5D3 was diluted 100-fold in RPMI 1640. The number of colonies in the control was 99 ± 6 for K562 and 104 ± 8 for HL60. (B) Virions were preincubated with different concentrations of ascites containing 5D3 before exposure to K562 and HL60 cells. The ROE was 3×10^4 virions per cell. The number of colonies in the control was 90 ± 2.5 for K562 and 81 ± 1 for HL60. (C) Recombinant and purified HBV-related antigens do not inhibit K562 colony formation. HBV, Serum containing HBV DNA so that the ROE was 10^5 virions per cell; HBsAg, recombinant small HBsAg at a concentration of 13 ng/cell; HBcAg, recombinant HBcAg; HBs/preS, large, middle, and small HBsAgs purified from serum; HBV DNA, serum-derived HBV DNA after proteinase K digestion, phenol-chloroform extraction, ethanol precipitation, reconstitution in RPMI 1640, and exposure to K562 cells at an ROE of 3×10^4 DNA molecules per cell. (D) Antibodies to envelope-related epitopes neutralize HBV-mediated inhibition of colony formation. The ROE was 10^5 virions per cell with a supernatant of HepG24 cells that contained HBV DNA. 5D3, Murine ascites containing the 5D3 monoclonal antibody to HBsAg diluted 10-fold in RPMI 1640; 5D3*, murine ascites containing the 5D3 monoclonal antibody to HBsAg diluted 10-fold in RPMI 1640 and incubated for 2 h at 37°C with purified HBsAg and pre-S antigens; Meas, culture supernatant for a murine hybidoma with antibody specificity for the measles virus; IgG, murine ascites from a monoclonal hybridoma with anti-human immunoglobulin specificity; D-preS, culture supernatant for a murine hybidoma with antibody specificity for the pre-S region of duck hepatitis virus (this antibody does not bind the pre-S and S regions of HBV); and WHs, murine ascites from a monoclonal hybridoma with anti-woodchuck hepatitis virus surface antigen specificity (this monoclonal antibody binds to the S region of HBV).

virions resulting from the various purification steps used to make the Dane particle preparation. The suppression of cell growth by purified virions and tissue culture-derived virus supports the hypothesis that the mechanism of inhibition involves a direct effect of the virus and not some other component found in serum. This hypothesis is also supported by our observations that antibodies to pre-S1, pre-S2, and S envelope proteins neutralized HBV inhibitory effects on colony formation. Since only viral particles contain pre-S1 sequences and since anti-pre-S1 antibodies neutralized the growth inhibition, these results imply that intact virus is involved in the inhibition of colony formation. This conclusion is also strengthened by the observation that incubation of anti-HBsAg monoclonal antibodies with HBsAg and pre-S antigens abolished the antibodies' ability to neutralize HBV-mediated inhibition of K562 colony formation. In addition, antibodies to epitopes unrelated to the envelope proteins of HBV (antibodies to duck hepatitis virus, measles virus, and human immunoglobulins) had no effect on HBV inhibition of colony formation. Only antibodies that cross-reacted with HBsAg neutralized the HBV effect.

Although we have previously found a direct effect of HBV on the growth and differentiation of bone marrow progenitor cells, we could not exclude that minor contamination of these cells with accessory cells somehow released substances that resulted in inhibition. The fact that HBV inhibits clonally derived hemopoietic cell lines implies that the effects of the virus are not accessory cell related. To date, our efforts to establish B-cell and T-cell cultures that contain

HBV DNA from the peripheral blood mononuclear cells of chronically HBV-infected patients have been unsuccessful. This failure might reflect the inhibitory effects of HBV on cell growth, as were observed in the hemopoietic cell lines.

In conclusion, hemopoietic cell lines may be used to study the relationships between HBV and abnormalities of hemopoiesis associated with viral hepatitis. This model may complement other recently described in vitro HBV culture models, including HBV-infected human B cell hybridomas (3), transfected hepatoma and hepatoblastoma cell lines (10, 11), and HBV-infected human hepatocytes (6).

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LITERATURE CITED

1. **Acs, G., M. A. Sells, R. H. Purcell, P. Price, R. Engle, M. Shapiro, and H. Popper.** 1987. Hepatitis B virus produced by transfected Hep G2 cells causes hepatitis in chimpanzees. Proc. Natl. Acad. Sci. USA **84**:4641–4644.
2. **Carmichael, J., W. G. De Graff, A. F. Gazdar, J. D. Minna, and J. B. Mitchell.** 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay. I. Assessment of chemosensitivity testing. II. Assessment of radioactivity. Cancer Res. **47**:936–946.
3. **Colucci, G., P. Lyons, Y. Beazer, and S. D. Waksal.** 1988. Production of hepatitis B virus-infected human B-cell hybridoma: transmission of the viral genome to normal lymphocytes in coculture. Virology **164**:238–244.
4. **Dejean, A., C. Lugassy, S. Zafrani, P. Tiollais, and C. Brechot.** 1984. Detection of hepatitis B virus DNA in pancreas, kidney and skin of two human carriers of the virus. J. Gen. Virol. **65**:651–655.
5. **Elfassi, E., J. L. Romet-Lomonne, M. Essex, M F. MacLane, and W. A. Haseltine.** 1984. Evidence of extrachromosomal forms of hepatitis B viral DNA in a bone marrow culture obtained from a patient recently infected with hepatitis B virus. Proc. Natl. Acad. Sci. USA **81**:3526–3528.
6. **Gripon, P., C. Diot, N. These, I. Fourel, O. Lorel, C. Brechot, and C. Guguen-Guillouzo.** 1988. Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. J. Virol. **62**:4136–4143.
7. **Lie Injo, L. E., M. Balasagaram, C. G. Lopez, and A. R. Herrera.** 1983. Hepatitis B virus DNA in liver and white blood cells of patients with hepatoma. DNA **2**:301–308.
8. **Pontisso, P., M. C. Poon, P. Tiollais, and C. Brechot.** 1984. Detection of HBV DNA in mononuclear blood cells. Br. Med. J. **288**:1563–1566.
9. **Romet-Lemonne, J. L., M. F. MacLane, E. Elfassi, W. A. Haseltine, J. Azocar, and M. Essex.** 1983. Hepatitis B virus infection in cultured human lymphoblastoid cells. Science **221**: 667–669.
10. **Sells, M. A., M. L. Chen, and G. Acs.** 1987. Production of hepatitis B virus particles in HEPG2 cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. USA **84**:1005–1009.
11. **Sureau, C., J. L. Romet Lemonne, J. I. Mullins, and M. Essex.** 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transection with cloned circular HBV DNA. Cell **47**:37–47.
12. **Wands, J. R., and V. R. Zurawski, Jr.** 1980. High-affinity monoclonal antibodies to hepatitis B surface antigen (HBsAg) produced by somatic cell hybridization. Gastroenterology **80**: 225–232.
13. **Zeldis, J. B., E. Ben-Porath, R. Enat, K. Kirsch, and J. Wands.** 1986. Correlation of HBV DNA and monoclonal reactivity to HBsAg in serum of patients with HBV infection. J. Virol. Methods **14**:153–166.
14. **Zeldis, J. B., F. A. Farraye, and H. N. Steinberg.** 1986. In vitro hepatitis B virus suppression of erythropoiesis is dependent on the multiplicity of infection and is reversible with anti-HBs antibodies. Hepatology **8**:755–759.
15. **Zeldis, J. B., H. Mugishima, H. Steinberg, E. Nir, and R. P. Gale.** 1986. HBV inhibition of myeloid differentiation. J. Clin. Invest. **78**:411–417.