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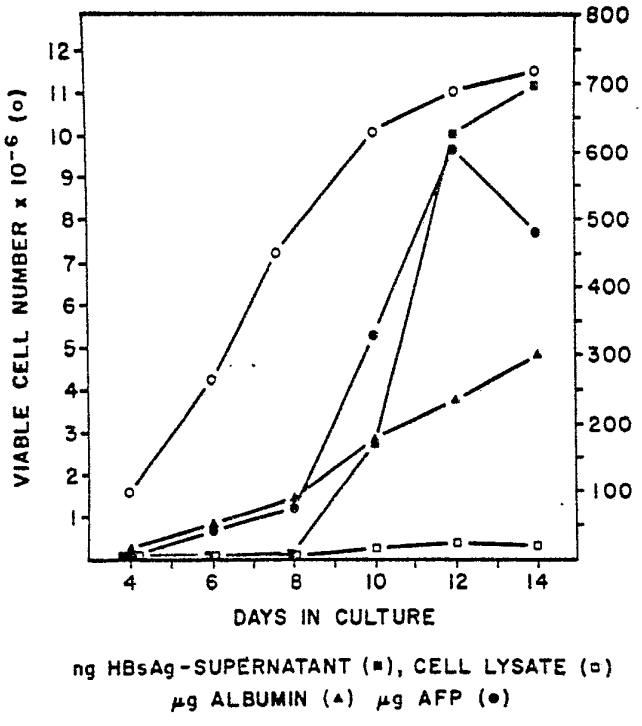
(54) Title: HUMAN HEPATOMA DERIVED CELL LINE, PROCESS FOR PREPARATION THEREOF, AND USES THEREFOR

(57) Abstract

Human hepatoma cell lines, useful for metabolic studies such as screening potential carcinogens and mutagens, for cultivation of viruses, and for preparation of vaccines, are obtained by culturing human hepatocarcinoma or hepatoblastoma on lethally irradiated cell feeder layers in the presence of a culture medium.



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Description

Human Hepatoma Derived Cell Line, Process for Preparation Thereof, and Uses Therefor

The invention described herein was made in the course of work under a grant or award from the Department of Health, Education, and Welfare.

Background of the Invention

There have been many attempts to develop cell culture systems for metabolic studies of chemicals, particularly for the short term assay of potential carcinogens and mutagens. The cell cultures in general use for such purposes are derived from rodents, and although they actively metabolize chemicals generally thought to be carcinogenic, the metabolites are different from those produced in normal human primary cultures. Human fibroblastic cell strains have been tested for their ability to convert potential carcinogens and mutagens to active carcinogens, but their ability to effect such metabolic conversions is low.

There are a number of problems associated with the growth of viruses for the production of vaccines. In particular, fastidious viruses, such as hepatitis B virus (HBV), have not been propagated successfully in cell cultures. Thus, cell culture systems capable of rapid growth which support production of viral components must be found in order for a vaccine to be produced from such fastidious viruses.

Objects of the Invention

A primary object of this invention is to produce stable hepatic cell lines useful for drug metabolism studies and particularly for screening potential carcinogens and mutagens.

Another primary object of the invention is to produce hepatic cell lines useful in the production of hepatitis B viral components from which vaccines can be made.

A further object of this invention is a process for derivation of human hepatic cell lines useful in the screening of drugs and especially potential carcinogens and mutagens, for cultivation of viruses, and for preparation of vaccines.

Still another object of the invention is a method for producing a hepatitis B vaccine employing the hepatic cell lines of this invention.

These and other objects of this invention will become further apparent from the following specification, appended claims, and accompanying drawings in which:

Figure 1 is a plot of time in culture (days) vs. viable cell numbers x 10^{-6}, and components of hepatitis B virus surface antigen (HBsAg), human albumin, and human α-fetoprotein (AFP) concentration in the cell active fluid, and

Figure 2 is an autoradiogram showing HBsAg in a control sample and in cell line Hep 3B of this invention, and the absence thereof in Hep G2 of said invention.

Detailed Description of the Invention

By the process of this invention there are produced novel stable cell lines suitable for use in metabolic studies, carcinogenesis and/or mutagenesis, and in the production of vaccines. The cell lines are obtained by culturing human hepatocarcinoma or hepatoblastoma on lethally irradiated cell feeder layers in the presence of a suitable culture medium. Although the present invention is applicable to the production of novel cell lines from any human hepatoma,

it is described in greater detail hereinbelow in particular with the production of two specific cell lines designated Hep G2 and Hep 3B. These cell lines have been deposited with the Wistar Institute of Anatomy and Biology, 36th and Spruce Streets, Philadelphia, Pennsylvania, 19104, and with the American Type Culture Collection (ATCC), Rockville, Maryland. Cell lines Hep G2 and Hep 3B have been assigned ATCC Nos. HB 8065 and HB 8064, respectively. Access to these two cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents to be entitled thereto under 37 CFR §1.14 and 35 USC §122, and all restrictions on availability to the public of the cultures so deposited will be irrevocably removed upon the granting of a patent.

Cell lines designated Hep G2 and Hep 3B were derived as a result of biopsies taken during extended lobectomies of a 15 year old caucasian male from Argentina (1975) and an 8 year old black male from the United States (1976), respectively. The cells from which the cell line designated Hep 3B was derived contained hepatitis B virus, as can be seen by reference to Figures 1 and 2, discussed below. However, this invention also contemplates infecting cells with HBV in order to produce HBsAg components for vaccine use.

Minces of the malignant tumors so obtained are cultured on lethally irradiated mouse cell layers designated STO in a cell culture medium consisting of Williams E medium (Gibco) supplemented with 10 percent fetal bovine serum (Reheis, Armour Pharmaceuticals). By the term "lethally irradiated" is meant that the cells have been irradiated to such a degree as to be incapable of replication. This procedure promotes the growth of differentiated cells with fastidious growth requirements while preventing overgrowth of contaminating fibroblastic cells.

The initial period of cell proliferation may take place over a period of at least about three weeks, and if desired may continue for many weeks. Ordinarily an initial proliferation period of about four weeks is quite satisfactory, following which cell colonies are separated and transferred to new irradiated mouse cell (STO) feeder layers. Before transfer, cells from flasks containing single large colonies may be dissociated by trypsinization (0.25% trypsin, 0.1% ethylenediamine tetraacetic acid in Dulbecco's modified phosphate buffered saline solution lacking calcium and magnesium salts). The cell colonies are subseqently serially passaged at least about sixty times on STO feeder layers showing that they are established, routinely growing cell lines, each passage having a duration of about one week.

Sublines of both Hep G2 and Hep 3B which no longer require the presence of the STO feeder layer were selected (four years and two years after establishment of the biopsies in the case of Hep G2 and Hep 3B, respectively). Such sublines will proliferate if approximately 1×10^6 cells are placed in 12 ml. of cell culture medium, such as Eagle's minimal essential medium supplemented with 10 percent of fetal bovine serum in a flask containing approximately 75 $cm.^2$ of growth area for the cells after attachment to the substratum. Although the above-described culture medium is preferred, other culture medium formulations may be used for supporting growth of these cell lines.

These two specific cell lines have been characterized as follows:

1. Chromosome number:

Hep G2 - the modal number of chromosomes is 55 (range 50-56). The cell line contains a marker chromosome which is a rearrangement of chromosome 1.

Hep 3B - the modal number is 60 with a subtetraploid mode of 82. This cell line contains a

marker chromosome which is a rearrangement of human chromosome 1.

2. Human Plasma Proteins:

The secreted products in the cell culture fluid of each cell line (Hep G2 and Hep 3B) have been characterized by both Ouchterlony double diffusion immuno-precipitation analysis (using commercially available antibodies to human plasma proteins), and by two-dimensional polyacrylamide gel electrophoresis, and are the following normal human plasma proteins:

Table I

Human Protein	Cell Line Hep G2	Hep 3B
α-fetoprotein	+	+
albumin	+	+
α-2-macroglobulin	+	+
α-1-antitrypsin	+	+
α-1-antichymotrypsin	+	+
transferrin	+	+
haptoglobin	+	+
ceruloplasmin	+	+
plasminogen	+	+
G_c globulin	-	+
Complement (C'3)	+	+
Complement (C'4)	+	+
C'3 activator	+	-
α-1-acid glycoprotein	+	+
fibrinogen	+	+
α-2-HS glycoprotein	+	+
retinoic acid binding protein	+	+
β-lipoprotein	+	+

3. Production of HBsAg:

Production of HBsAg has been quantitated in the Hep 3B cell line using the AUSRIA II (Abbott Labs)

solid phase radioassay (RIA) kit comparing positive values to a standard curve using purified HBsAg. See Figure 1, in which the curve having points in the form of solid squares represents ng of HBsAg in cell culture supernatant vs. time, the curve having open square points represents ng HBsAg in cell lysate, and the curve having solid triangular points represents µg human albumin. The remaining curves having points in the form of solid and open ovals represent, respectively, µg of alpha-fetoprotein and viable cell number beginning with 10^6 Hep 3B cells in 15 ml. Eagle's minimal essential medium supplemented with 10 percent fetal bovine serum.

The components of HBsAg synthesized by Hep 3B were determined by pulse labeling the cells by exposure for 5 hours to 5 ml. Eagle's minimal essential medium free of fetal bovine serum, but containing 1 mCi of ^{35}S-methionine (New England Nuclear Company) and incubating 1.5 ml. of ^{35}S-methionine labeled cell supernatant with 10 microliters of guinea pig anti-HBsAg (obtained from National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Maryland, 20014), and then with 20 λ rabbit antiserum to guinea pig immunoglobulin G. After an overnight incubation, immune complexes were obtained by centrifugation, washed and resuspended in a solution containing 1 M dithiothreitol, 2% sodium dodecylsulfate and subjected to SDS polyacrylamide gel electrophoresis on a 7.5-15 percent linear gradient gel, dried, and exposed to Kodak NST2 film. The results obtained are illustrated in the autoradiogram of Figure 2, by reference to which it can be seen that p 23 and p 27 components of HBsAg are obtained from the sample of Hep 3B (see gel lane 2). These components are also present in the control sample containing purified HBsAg (gel lane 1), but are absent from Hep G2, which was not infected with HBV (gel lane 3).

The production of HBsAg components by cell line Hep 3B, which contains the hepatitis B virus genome, shows that these components can be purified for use in vaccine production, or that by infecting hepatic cell lines produced by the process of this invention, similar components could be obtained for use in providing an HBV vaccine.

The following non-limiting examples are illustrative of various embodiments of this invention.

Example I

This example illustrates the use of the cell line of this invention designated Hep G2 for metabolic studies, in particular for screening possible carcinogens and mutagens.

Confluent cultures of Hep G2 cells were exposed to 4.0 n moles/ml. of ^{3}H-benzo(a)pyrene (BP) in medium for 24 hours; most of the radioactivity was recovered in the medium. A significant portion of the BP was metabolized to water-soluble metabolites which, after treatment with β-glucuronidase, yielded chloroform-extractable BP-quinones and 3-OH-BP. Of the chloroform-extractable material in the Hep G2 cell culture, the following metabolites were formed:

Table II

Metabolite	Percent Metabolites Based on Total BP
BP 9,10 diol	14
BP 7,8 diol	7
Quinones	6

Thus, the Hep G2 cell line metabolizes BP to a number of oxidized derivations, including the proximate carcinogenic metabolite BP 7,8 diol.

Studies of the BP-DNA adducts formed in the Hep G2 cells yielded the following results. Of 4.0 n

moles of ^{3}H-BP/ml. of medium with 48 hour exposure, over 97 percent was metabolized. The DNA isolated from these cultures contained 28.2 p moles of bound BP/mg. DNA. The BP-DNA adducts resemble those formed in primary organ cultures from human tissue. Since it is generally accepted that polycyclic aromatic hydrocarbons, and many other classes of carcinogens, require metabolic activation to produce their biological effects and the Hep G2 cells metabolize these compounds to the activated form, the Hep G2 cells can be used as activators of carcinogens in a cell mediated mutation assay with other mammalian cells.

Example II

Hep 3B was exposed to 0.5 n moles ^{3}H-BP/ml. in medium for 24 hours, and 76 percent of the ^{3}H-BP was metabolized to water-soluble intermediates. Of the chloroform-extracted material, 76 percent was unchanged ^{3}H-BP, but small amounts of the BP 9,10 diol and the BP 7,8 diol were detected.

As indicated previously, the cell lines of this invention should be useful in the cultivation of viruses, particularly fastidious viruses such as HBV. In such use, monolayer cultures of the particular cell line, e.g. Hep G2, may be exposed either to Dane particles from infected patients' sera, or to HBV-DNA purified from such a source. After about an hour of absorption to the monolayer in small amounts of the culture medium, additional cell line culture medium may be added and the flasks containing the cell line incubated for several days. Monitoring of the cultures by standard techniques for detection of viral antigens, such as described above in connection with cell line Hep 3B, will determine the optimum time for harvest of cell cultures and virus antigen purification. The antigens so produced, e.g. HBsAg, may be used for production of vaccines.

Where the initial hepatocarcinoma used in the process of this invention already contains the HBV genome, such as is the case with the source material from which the Hep 3B cell line was obtained, the HBsAg synthesized by the cell line can be used to produce a vaccine.

Advantageously, a cell line of this invention containing HBV, e.g. Hep 3B, provides an alternate source of HBsAg whereby the production and quality of the antigen may be controlled. Experiments with Hep 3B indicate that only a portion of the viral genome is present, and that infectious viral particles are not produced by this cell line, thereby eliminating the risk of HBV infection. Purification of the HBsAg from the cell culture medium for vaccine production can be effected by a number of well-known methods in the art.

An alternative method of preparation of HBsAg from the cell lines of this invention involves the use of recombinant HBV-plasmid DNA in an in vitro HBsAg synthesizing bacterium. To prepare a vaccine employing this technique, the total cellular RNA, from which cDNA copies are made, or DNA from the HBsAg-producing cell line is isolated and digested with restriction enzymes that do not digest the HBV-DNA or leave the HBsAg coding segment intact (e.g. Hind III), followed by enrichment of the HBV-DNA, either by hybridization to filters containing fixed Dane particle DNA and elution from the filters, or by elution from electrophorograms after localization to a portion of the gel with radioactively labeled Dane particle derived DNA. After reaction with dCMP residues, using nucleotidyl-terminal transferase, the DNA can be hybridized to plasmid pBR322, cleaved with PST-1 and tailed with d GMP residues, and E. coli can be transformed with hybrid plasmids and screened for HBsAg-producing colonies by methods well-known in the art. HBsAg positive clones can then be selected, propagated and vaccine production from these clones would

proceed (Wu, R., editor; Methods in Enzymology, Volume 68, Academic Press, N. Y. for overall recombinant DNA procedure).

Claims

1. A process for the derivation of a human hepatic cell line which comprises culturing a biopsy of a human hepatic tumor on lethally irradiated cell feeder layers in the presence of a culture medium until a cell line is established.

2. A process according to claim 1 in which said irradiated cell feeder layers comprise mouse cell layers.

3. The process according to claim 1 in which said tumor is initially cultured on lethally irradiated feeder layers for at least about three weeks to cause cell proliferation, and the resulting cell colonies are subsequently serially passaged at least sixty times on said feeder layers to establish a cell line.

4. A process for assessing the metabolic conversion of certain chemicals and drugs, particularly potential carcinogens and mutagens, which comprises:

(a) maintaining a culture of a human hepatic cell line in a nutrient medium,

(b) exposing said cell line to the chemical or drug to be tested,

(c) analyzing said culture for the presence of metabolites of said chemical or drug, and

(d) introducing said metabolites to cultures of other mammalian cells to determine their mutagenic capabilities.

5. The process according to claim 4 in which said cell line is produced by the process of claim 1.

6. The process according to claim 5 in which said cell line comprises Hep G2.

7. The process according to claim 5 in which said cell line comprises Hep 3B.

8. A process for the cultivation of a fastidious virus, such as hepatitis B virus, which comprises:
   (a) maintaining a culture of a human hepatic cell line in nutrient culture medium,
   (b) inoculating said medium with a fastidious virus,
   (c) cultivating said virus in said cells of said cell line, and
   (d) recovering a harvest of said virus from said cell line.

9. The process of claim 8 in which said cell line is produced by the process of claim 1.

10. The process according to claim 9 in which said cell line comprises Hep G2.

11. The process according to claim 8 in which said fastidious virus is hepatitis B virus.

12. A process for isolation of hepatitis B virus surface antigens for use as a vaccine which comprises:
   (a) maintaining a human hepatic cell line which contains the hepatitis B virus genome in nutrient culture medium,
   (b) recovering the supernatant fluid from said culture, and
   (c) purifying the hepatitis B virus surface antigen in said supernatant fluid for use as a vaccine.

13. The process according to claim 12 in which said cell line is produced by the process of claim 1.

14. The process according to claim 13 in which said cell line comprises Hep 3B.

15. A process for producing hepatitis B virus surface antigens from a human hepatic cell line for use in a vaccine which comprises:

(a) obtaining DNA or RNA from a human hepatic cell line containing the hepatitis B virus genome,

(b) producing recombinant DNA plasmids containing the hepatitis B viral genome obtained from said DNA or RNA,

(c) growing said recombinant DNA plasmids in a bacterial host, and

(d) purifying and recovering hepatitis B virus surface antigen for use as a vaccine.

16. The process according to claim 15 in which said cell line is produced by the process of claim 1.

17. The process according to claim 16 in which said cell line comprises Hep 3B.

18. A process for isolation of human plasma proteins for use in the treatment of patients deficient in said proteins which comprises:

(a) maintaining a human hepatic cell line which synthesizes the desired plasma proteins in a nutrient culture medium,

(b) recovering the supernatant fluid from said culture, and

(c) purifying said plasma protein for use in treatment of patients deficient in said protein.

19. The process according to claim 18 in which said cell line is produced by the process of claim 1.

20. The process according to claim 19 in which said cell line comprises Hep G2.

21. The process according to claim 19 in which said cell line comprises Hep 3B.

22. A process for producing human plasma proteins for use in treatment of patients deficient in said proteins which comprises:

(a) obtaining DNA and/or RNA from a human hepatic cell line which synthesizes the desired plasma proteins,

(b) producing recombinant DNA plasmids containing the cDNA for the relevant proteins,

(c) growing said recombinant DNA plasmids in a bacterial host, and

(d) purifying and recovering the desired human proteins for use in treatment of humans deficient in said proteins.

23. The process according to claim 22 in which said human hepatic cell line is obtained by the process of claim 1.

24. The process according to claim 23 in which said cell line comprises Hep G2.

25. The process according to claim 23 in which said cell line comprises Hep 3B.

26. A cell line produced by the process of claim 1.

27. The cell line having the identifying characteristics of Hep 3B.

28. The cell line having the identifying characteristics of Hep G2.

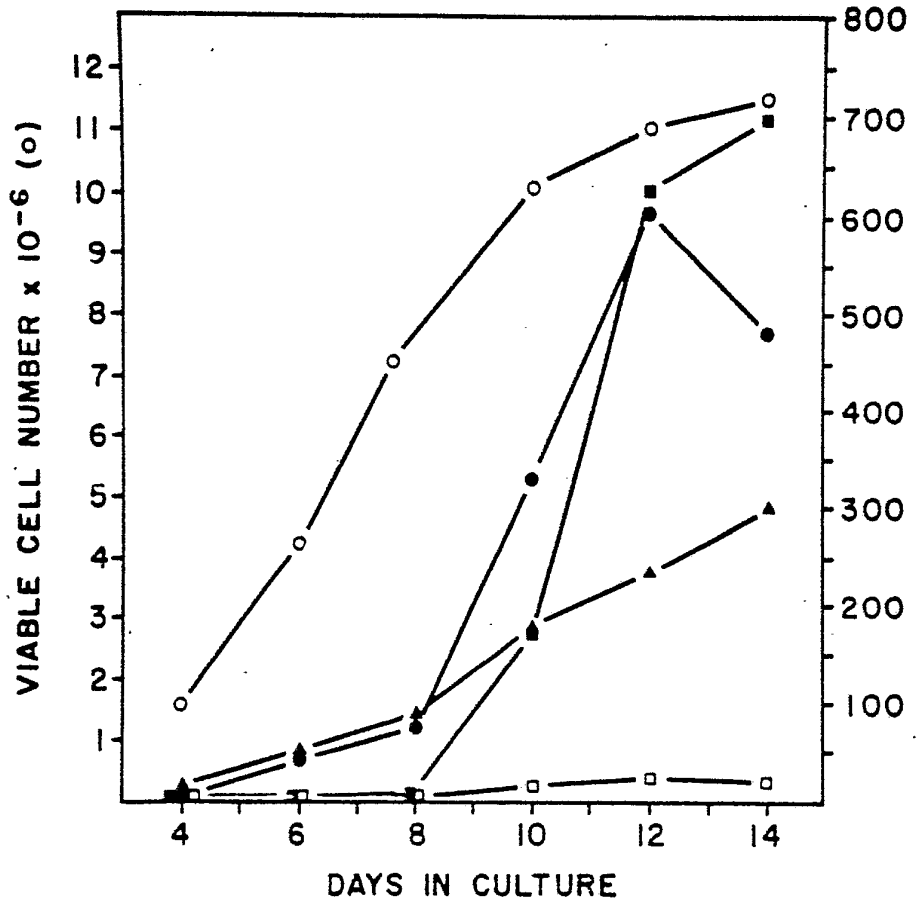

FIG. 1

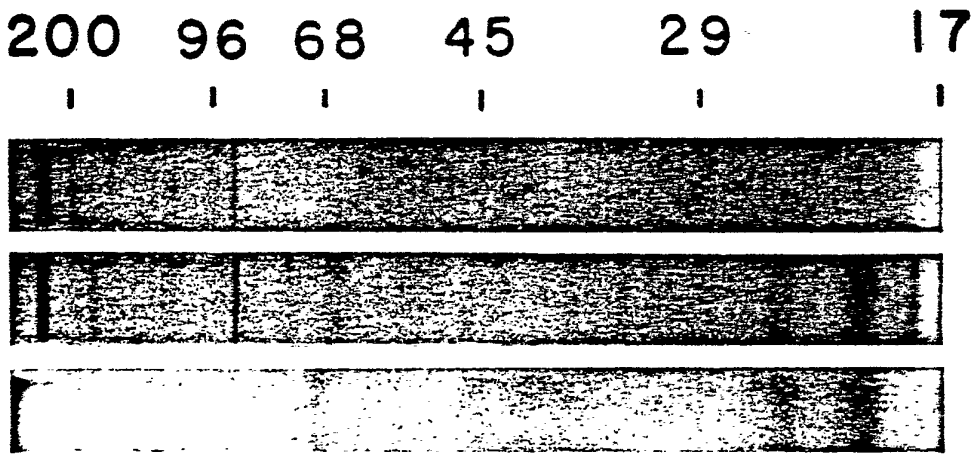

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US81/00778

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL. C12N 7/00,15/00 ,,5/00; C12R 1/91
U.S. CL. 435/68,172,235,240,948

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68,172,235,239,240,241,948; 424/12,86,89

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CHEMICAL ABSTRACTS, VOLUMES 76-92 (1972-1980)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X,A	NATURE, 282, ISSUED 06 DECEMBER 1979, DAVID P. ADEN, "CONTROLLED SYNTHESIS OF HBsAg IN A DIFFERENTIATED HUMAN LIVER CARCINOMA-DERIVED CELL LINE", PAGES 615-616.	1-3,6,7,10-14, 17,20,21,24-28
A	METHODS IN ENZYMOLOGY, VOLUME LVIII, ISSUED 1979, LOLA C.M. REID, "CLONING", PAGES 152,153,162-164.	1-3,5,9,16,19, 23
A	CHEMICAL ABSTRACTS, 91:155286a, ISSUED 1979, ROBERT LANGENBACH, "MAINTENANCE OF ADULT RAT HEPATOCYTES ON C3H/10T 1/2 CELLS", PAGE 433.	1-3,5,9,16,19, 23
A	JUNTENDO IGAKU, 24(3), ISSUED 1978, MASANORI IWADARE, "EFFECT OF DRUGS ON CULTURED LIVER CELLS", PAGES 307-313.	4-7
A	BRITISH JOURNAL OF CANCER, 34, ISSUED 1976, G.M. McNAB ET AL, "HEPATITIS B SURFACE ANTIGEN PRODUCED BY A HUMAN HEPATOMA CELL LINE", PAGES 509-515.	12-14
A	US,A, 3,871,954, PUBLISHED 18 MARCH 1975, ZUCKERMAN.	8-11
A	NATURE, 279, ISSUED 03 MAY 1979, C.J. BURRELL ET AL, "EXPRESSION IN ESCHERICHIA COLI OF HEPATITIS B VIRUS DNA SEQUENCES CLONED IN PLASMID pBR 322", PAGES 43-47.	15-17
A	GB,A, 2,034,323, PUBLISHED 04 JUNE 1980, TIOLLAIS ET AL.	15-17

* Special categories of cited documents: [15]

"A" document defining the general state of the art

"E" earlier document but published on or after the international filing date

"L" document cited for special reason other than those referred to in the other categories

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but on or after the priority date claimed

"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
21 AUGUST 1981	03 SEP 1981
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Esther M. Kepplinger ESTHER M. KEPPLINGER

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	CHEMICAL ABSTRACTS, 90:201941q, ISSUED 1979, YU. T. ALEKSANYAN, "PRODUCTION OF SERUM PROTEINS BY HEPATOMA XXII A CELL CULTURES", PAGE 446.	18-21
A,P	US,A, 4,209,587, PUBLISHED 24 JUNE 1980, TOLBERT ET AL.	18-21
A,P	US,A, 4,237,224, PUBLISHED 02 DECEMBER 1980, COHEN ET AL.	22-25
A	US,A, 4,164,566, PUBLISHED 14 AUGUST 1979, PROVOST ET AL.	8-11
A	SOUTH AFRICAN JOURNAL OF MEDICAL SCIENCES, 41, ISSUED 1976, JENNIFER ALEXANDER ET AL, "ADAPTATION OF CELLS DERIVED FROM HUMAN MALIGNANT TUMORS TO GROWTH IN VITRO", PAGES 89-98.	26-28

CONTINUED ON EXTRA SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE USA, 72(4), ISSUED APRIL 1975, GAIL R. MARTIN ET AL, "DIFFERENTIATION OF CLONAL LINES OF TERATOCARCINOMA CELLS: FORMATION OF EMBRYOID BODIES IN VITRO", PAGES 1441-1445.	12-14