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(54) Title: MONOCYTOID CELL LINE, ITS PRODUCTION AND METHOD FOR INFECTION WITH VIRUS, ESPECIALLY HIV

(57) Abstract

Cell line of monocytoid cells capable of unlimited growth *in vitro*, characterized by being a subclone of a monocytoid wild type. Method of producing virus particles, in the first place HIV, or fragments thereof by infecting one of the aforesaid cell lines which has a T4 content exceeding 50 %. Method of producing the cell lines, comprising cloning and selection of the clones which have a T4 content exceeding 5 %.

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Monocytoid cellline, its production and method for infection with virus, especially HIV.

The invention is concerned with novel cell lines adapted especially for culturing and studying retroviruses capable of infecting the cells of these cell lines. Among the retroviruses contemplated may be mentioned HIV (Human Immuno Deficiency Virus). Various isolates of HIV have previously been designated LAV (Lymphoadenopathy Associated Virus), HTLV-III (Human T Lymphotropic Virus III) and ARV (AIDS-associated Retrovirus).

In the case of AIDS (acquired immune deficiency syndrome) it appears that the principal immunological abnormality resides in a quantitative defect in the helper T cell subset expressing the T4 antigen on the cell surface. A selective T4 cell tropism of various HIV isolates has been demonstrated in vitro. Virus replication and high reverse transcriptase activity have been observed in populations of T4 cells but not in the corresponding T8 cells (11). Blocking experiments with monoclonal antibodies to the T4 and T4A antigens (12, 15) and inhibition of syncytia formation of VSV (HTLV-III) pseudotype virus (3) have indicated that the T4 antigen or a part thereof constitutes an important component of the HIV receptor.

Data from some laboratories, however, show that the HIV virus is also able to infect some cell types of non-T lymphoid lineage. Hybridizations have revealed the presence of proviral DNA in brain cells of AIDS patients (26). Electron microscopy has shown the presence of virus particles in follicular dendritic cells (30) and in macrophages (6). Virus has been isolated from peripheral blood monocytes (9) and alveolar lung macrophages of HIV infected individuals. Infection in vitro of monocytes by HIV has been described (34).

Infection in vitro with various HIV isolates has been reported in the case of (a) a malignant glioma cell line (2) and (b) B cells carrying the Epstein-Barr virus genome (16, 22). The infection is evidently latent in glioma

cultures while B cells allow virus replication. Furthermore, the promyelocytic cell line HL-60 and the monocytic line U-937 (ATCC CRL 1593) have also been infected with the ARV isolate (13). Productive HTLV-III infections of normal peripheral blood monocytes have been demonstrated repeatedly (4, 9). Within a short time interval the spectrum of cell types that can be infected by HIV has been broadened.

For culturing HIV in vitro it has been proposed in the patent literature that (i) a neoplastic aneuploid T cell line (33) and (ii) a lymphoblastoid B cell line (32) should be employed. As regards (i) the subclone H9 has been mentioned as a preferred cell line.

In an International Type Search Report the Swedish Patent Office has cited references 3, 13 and 34 as being of particular relevance. References 9, 33, 35 and 36 have been cited as defining the general state of the art.

The object of this invention is to provide means for promoting the understanding of diseases caused by viruses which are capable of infecting monocytoid cells. Among viruses contemplated in this context may be noted in particular certain retroviruses such as HIV. The invention thus provides cell lines (cell populations) the cells of which can be infected in vitro by the virus in question and consequently are potentially utilizable also for virus production in vitro, and furthermore for studying i.a. the AIDS pathogenetic mechanism on a cellular level and for studying the manner in which drugs will affect the course of the disease. A first aspect of the invention comprises the cell lines as such, a second aspect comprises the development of said cell lines, and a third aspect comprises the use of said cell lines for in vitro infection and optionally large-scale production of the virus. As a further aspect may be mentioned the virus-infected cell line in itself which may be an excellent diagnostic tool (e.g. in radioimmunoprecipitation).

The cell line of the invention is a stable subclone of a monocytoid wild type and is capable of unlimited growth in vitro. The cell lines are characterized in that T4 antigens are expressed on the surface of at least about 5 %, e.g. more than 10 %, of the cells of the population. The preferred forms thereof can be infected productively. Other characteristic features reside in that class II antigen and Fc receptors may be found present as cell surface antigens on at least some of the cells of the population. These characteristics can be demonstrated if the cell lines of the invention are studied under the conditions set forth in the experimental portion of this application.

The cell lines of the invention are potentially obtainable by cloning monocytoid cell lines which are derived from cancer tissue and are capable of unlimited growth. As an example may be mentioned U-937 which is of histiocytic origin. It was described for the first time by Sundström and Nilsson (28) and has been deposited at the ATCC as No. ATCC CRL 1593. Examples of the cell lines of the invention (clone 1, 2 and 16) have been deposited at the European Collection for Animal Cell Cultures, Salisbury, England; they have been given the preliminary designations of clone 1 86121901, clone 2 86121902 and clone 16 86121903. Redeposits have been made and given the designations 87100805, 87100806 and 87100807, respectively. They have been accepted to be in accordance with the Budapest Treaty of 1977. U-937 is cytogenetically abnormal. Studies of its karyotype have shown that its chromosomal alterations are progressive. Detailed studies of the chromosome complements in the cell lines of the invention are currently under way and are expected to show that every clone has a distinct karyotype.

The cloning technique employed is known per se. We have conducted cloning successfully in agar gel. Potentially other techniques too may be employed, such as the so-called limiting dilution technique where the clone cells have been allowed to grow independently of one another. After cloning the proliferating clones fulfilling the characteristics of the present invention are selected. The scope of the invention

of course comprises also such cell lines as are obtained upon recloning of subclones that have been obtained in a first cloning of the wild type.

Most of the clones obtainable from the preferred wild type U-937 will form stably virus-producing lines after viral infection - thus differing from H9 and other T cell lines which show much variation in respect of their virus production. The clones preferred according to the invention, that is, those having a high T4 content (e.g. clone 16) may have all the properties inherent in a "classical" virus-cell line where the viral infection results in the production of a large amount of virus, with subsequent death of the cells (=cytopathogenic effect). As a consequence it is possible to predict when the large amounts of virus will be released; this is an important prerequisite for large-scale production. Thus immediately before their death the cells are converted to "virus factories" with a large amount of viral antigen which can easily be tagged by isotope labeling and then be used in radioimmunoprecipitation procedures.

In the cell lines of the invention, the percent amount of cells possessing T4 antigen (= T4 content) may vary from as low as 5 % up to 100 % or just below 100 %, e.g. 95 %. The cell lines preferred for virus production, i.e. those giving a productive infection, will preferably have a T4 content exceeding 50 %, as e.g. exceeding 60 or 70 % although cell lines expressing lower T4-content may also be used.

The cell lines of the invention are cultured in vitro in a medium such as is commonly employed for U-937 or in some other medium resulting in satisfactory growth. Infection of the lines is performed in a manner such as in common practice in the case of each particular virus employed, the cell line being exposed to a suitable virus inoculum, for instance HIV. In some cases a practical expedient may reside in that, some days after the addition of the first virus inoculum to

the cell line, fresh cells are added which are uninfected and have properties as according to the present invention. Cell lines having a T4 content of less than about 50-60 % are suitable in the first place for studies of the virus-cell interaction. From virus-producing cell lines it is possible in a manner known per se to isolate the virus, purify it and if desired also subject it to fragmentation and derivatization. The virus products obtained may be used for developing drugs for the treatment and prevention of the corresponding virus infection, e.g. vaccines, and/or for developing reagents for immunochemically assaying the virus and/or corresponding homologous antibodies.

The invention is defined in the attached claims which form an integral part of the specification.

The invention will now be further illustrated by way of the scientific work underlying the invention.

Example 1

Materials and Methods

Cells: The U-937 cell line derived from a histiocytic lymphoma (28) was grown in RPMI 1640 or F-10 supplemented with 10 % fetal on new born calf serum and antibiotics (penicillin 100 IU/ml, streptomycin 50/ug/ml). The cell line has been kept in continuous passage since its establishment in 1976 and has retained the basic phenotype corresponding to that of an immature monocyte. The cell line is inducible by various agents to phenotypic alterations similar to those of normal monoblasts undergoing differentiation (5, 19, 20). U-937 thus expresses monocytic cell markers with a high degree of accuracy also when induced to further differentiation, and therefore appears to be a very useful model for human monocytic cells (19, 20).

Establishment of U-937 clones by agarose cloning: Cells (1×10^3) were spread evenly in 35 mm petri dishes (Falcon, Oxnard, Ca, USA). The dishes contained a bottom layer of Ham's F-10 together with 10 % FCS (GIBCO EUROPE, United Kingdom), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (150 µg/ml) and a final concentration of 0,5 % Agarose A (Pharmacia AB, Uppsala, Sweden).

The top layer (1 ml) in which the cells were spread out contained the same medium but with a final concentration of agarose of 0.33 %. The petri dishes were incubated for 2-4 weeks in a humified atmosphere (5 % CO_2 in air) at 37°C. During this period the petri dishes were observed for clonal growth of U-937 cells. Suitable clones were picked and the cells were transferred into 96-well plates and later into 24-well plates (Falcon) for further expansion. Two experiments yielded a total of 16 clones.

Five clones (clones 1, 2, 3, 4 and 16) showing differences inter se in T4 antigen expression were selected and further characterized with respect to a number of other markers. T4 and HLA-DR antigens were studied by flow cytometry using monoclonal antibodies directed against the antigens in question (Becton-Dickinson, Monoclonal Center, Mountain View, Ca. (10)). Fc receptor expression was determined using IgG coated erythrocytes. Selected cytoplasmic enzyme markers were studied by cytochemical techniques (29).

Virus infection: Cell-free medium from an H9 cell culture infected with HTLV-IIIB was used as the source of virus. 1×10^6 cells were resuspended in 1 ml of virus, incubated for 60 minutes at 37°C and resuspended in 5 ml of 10 % RPMI medium with 2 µg Polybrene (PB = 1,5-dimethyl-1,5-diazaundecamethylene-polymethobromide, Sigma, USA) and antibiotics.

Blocking of infection with the aid of monoclonal anti-T4 antibody: The IgG monoclonal anti-T4 antibody (19B5D7) employed had been provided by Dr Ellis Reinherz, Boston, Mass. Cells (2×10^5) were incubated with 2.5 µg or 12.5 µg anti-T4 antibody in a total volume of 75 µg RPMI medium for 20 minutes at 37°C and washed once with said medium. The cells were then resuspended in 125 µl of virus dilutions and incubated at 37°C for 60 minutes. Following incubation the cells were washed once more with the medium and re-suspended in 10 % RPMI medium containing 2 µg/ml PB at a concentration of 10^5 cells/ml, and cultured in 24-well plates. Anti-T4 antibody at a concentration of 1.25 µg/ml and 6.25 µg/ml, respectively, was present for the first 4 days but removed later. The medium was harvested twice a week for reverse transcriptase (RT) determination.

Determination of reverse transcriptase (RT): Enzyme activity was measured in a solution obtained by ultracentrifugation of the culturing medium and dissolution of the thus resultant pellet in 0.3 % Triton X-100. The reaction mixture (100 µl) contained 50 mM Tris pH 8.0, 160 mM KCl, 4 mM dithiothreitol, 6.2 mM $MgCl_2$, 25 µCi/ml ^{3}H-TTP (50 Ci/mmol), 100 µg/ml bovine serum albumin (BSA), 2.5 µg/ml oligo-dT_{12-18} and 2 µg/ml poly A.

Southern blotting (27): 10 mg of chromosomal DNA were isolated, cleaved with Sac I, electrophoresed on 0.8 % agarose gel, blotted onto nylon membranes and hybridized with a ^{32}P labeled probe. Cytoplasmic DNA was prepared using the Hirt procedure (8), normalized to mitochondrial DNA and run on 0.8 % agarose gel, then transferred to nylon membranes, and hybridized with a ^{32}P labeled probe. Hybridizations were done under stringent conditions using 3 x SSC (175.3 g NaCl and 88.2 Na citrate per liter of water, pH 7) at 68°C and 5×10^6 cpm/ml of labeled probe. Washing conditions were 0.1 x SSC at 68°C for all blots. The probe employed was pBH10-R3 (7) which had been provided by Dr R C Gallo (NIH, Bethesda, USA).

Results

Cell marker profiles of the parental cell line and subclones: The original cell line U-937 and its subclones were different in respect of their relative expressions of HLA-DR, T4 antigen, Fc receptors and enzymes (Table 1). It is particularly worth noting that the parental line (wild type), clone 4 and clone 16 represent extremes with respect to T4 expression. The parental line and clone 4 cultures contained T4-positive cells in an amount of less than 10 % whereas 95 % of the clone 16 cells were T4-positive. All other clonal lines assumed an intermediary position with 50-70 % T4-positive cells.

HLA-DR and Fc receptors showed a tendency for co-expression. The frequency of cells positive for both HLA-DR and Fc receptors was highest in clones 1 and 2, whereas both markers were less frequent in the parental cell line U-937 and clone 16 populations. With respect to growth properties, morphology, phagocytic activity, capacity of functioning as killer cells in antibody-dependent cellular cytotoxicity assays and expression of the monocyte-associated surface antigens defined by the M3 and OKM1 monoclonal antibodies no differences could be demonstrated between the cell lines studied under the culture conditions employed.

Infection of parental line and subclones with HTLV-IIIB: All cell lines could be infected with the HTLV-IIIB isolate (Table 2). However, the lines showed great differences with respect to the time required for virus production and in respect of their sensitivity to the cytopathic effect of the virus (syncytia formation and cell death).

The parental U-937 and clone 4 showed a similar pattern. No RT activity was detected in culture fluids of U-937 during the first six weeks following virus infection. In fact the cultures became RT-positive only two months after infection and then remained positive during the entire period of continued culturing (more than one year).

Infection of clone 4 cells was more difficult than infection of the parental line cells since the dose of virus that resulted in a virus-producing infection of all other cell lines (150 x 10^3 cpm in RT activity) failed to productively infect clone 4. During the entire observation period (three months) clone 4 remained RT-negative. However, a ten times higher dose of virus (RT activity 1800 x 10^3 cpm) yielded a virus producer culture within one week. Clone 4 cells were thus more difficult to infect than the parental line but this difficulty could be overcome by a higher dose of virus for infection. The parental line and clone 4 were similar in that no or only a marginal cytopathic effect with occasional syncytia formation could be observed in infected cultures.

Among the U-937 subclones, clone 16 was the most sensitive to infection with HTLV-IIIB. RT activity and cytopathic effect were demonstrable already six days after infection. Virus replication and cell lysis were however so pronounced that the cultures died out entirely. It was not possible to obtain virus producing cell lines even when fresh uninfected cells were repeatedly added to the infected culture. With respect to sensitivity to HTLV-IIIB infection, clones 1, 2 and 3 represented intermediates between the two extremes i.e. the parental line and clone 16. In cultures of clone 2 cells the cytopathic effect and RT activity appeared within 10 days after infection. Unlike clone 16, addition of uninfected clone 2 cells led to the establishment of a stable virus producer cell line one month later. Clone 2 has during the priority year been recloned and one of the cell lines then obtained (clone 2a) is an even better producer of HIV, showing a minimum of cytopathic effect. Clones 1 and 3 were less sensitive than clone 2 to the cytopathic effect of the virus. A transient cytopathic effect was observed three to four weeks after infection when the cultures were already producing virus (RT-positive culture broths. In the case of clone 1 addition of fresh uninfected cells was not necessary for establishing a stably virus producing cell line, whereas clone 3 did require such

an addition in order to compensate for cell death. All lines except clone 16, have remained stable virus producers for periods extending from six months and up to more than a year. None of them have shown any cytopathic effect during this period.

Presence and state of HTLV-IIIB DNA in U-937 parental cells and subclones: Southern blot analyses of Hirt lysates showed that all infected cell lines contained unintegrated proviral HTLV-IIIB DNA of the linear form. Some cell lines (clones 16 and 4) moreover contained still another type of proviral DNA, probably superhelical (supercoils) composed of unit lengths of circular DNA (unit length circles). Several faint bands, in addition to the expected bands, could be seen; they appeared only after a prolonged period of exposure. Presumably they represent defective forms or variants of the HTLV-IIIB provirus.

The cell lines differed in the number of free genome copies present in the cytoplasm of infected cells. An estimation of the number of copies could be made in that serial dilutions of the 9 kb fragment inserted by means of SacI into pBHI0-R3 were compared with the high molecular SacI fragments of the infected cells or with Hirt lysate DNA. Clone 16 cells contained approximately 100 copies per cell whereas the parental line contained about one copy per cell, as did the T cell positive control (H9). The other cell lines were intermediate with respect to their contents of copies. The highest molecular weight band (> 12 kb) probably represented HTLV-IIIB DNA that had penetrated into the mitochondria. Proviral DNA could not be demonstrated to be present in uninfected clone 16 cells.

High molecular DNA that had been digested with SacI showed mainly two bands representing DNA integrated from HTLV-IIIB. Each cell line that had been infected contained approximately one to five integrated copies of proviral DNA from HTLV-IIIB. Two fragments of 5.5 and 3.5 kb resp. corresponded to proviral DNA containing an internal SacI site as well as

SacI sites from proviral LTRs. All these bands were to be found also in the infected T cell line employed as control. (Molt-3). The uninfected clone 16 cells were negative with respect to the integrated form of proviral DNA.

Blocking of HTLV-IIIB infection by anti-T4 monoclonal antibody: Clone 16 was chosen for blocking experiments because of its high proportion of T4 positive cells and its extreme sensitivity to lysis by HTLV-IIIB. The H9 cell line served as a T cell control. The results of two experiments are shown in Table 3. For obtaining the blocking effect it was very important that virus infection followed immediately after treatment of the cells with antibodies. The H9 cells started to produce virus 32 days after infection with a 1:100 dilution of virus. Preincubation of the cells with 12.5 µg of anti-T4 antibody blocked infection completely. 2.5 µg of antibody on the other hand did not have any effect. If clone 16 cell cultures were infected with the same dose as that employed for the H9 cells (1:100 dilution) they produced virus already on day 15. Larger virus doses (1:100 dilution or undiluted) gave rise to virus production already on day 8. With 12.5 µg of anti-T4 antibody, infection with undiluted virus could only be delayed without being blocked completely; whereas infection with lower doses of virus could be blocked completely even with 2.5 µg of antibody. These results thus show that the epitope recognized by this particular antibody is important for the recognition of the cell surface by the virus.

Example 2

Radioimmunoprecipitation assay (RIPA): Antibodies to virus-related proteins were identified by RIPA (the antigen was ^{35}S-cysteine labeled lysate from infected cells)(17). Clone 16 and the human T cell line HUT-78 were used for preparation of antigen. Clone 16 (10^7 cells) was thus infected with HTLV-IIIB which had been obtained from R C Gallo (reverse transcriptase activity was 300 x 10^3 cpm). The infected

cultures were monitored for cytopathic changes, reverse transcriptase activity (1) and viral antigen expression by means of immunofluorescence with monoclonal antibodies directed against p24 and p19 which in turn were detected by rabbit anti-mouse IgG labeled with fluorescein isothiocyanate (Dakopatts, Glostrup, Denmark). The cultures were isotope labeled 7 days after infection when a pronounced cytopathic effect was evident and about 90 % of the cells expressed viral antigen.

After starvation in the labeling medium (cysteine-free RPMI 1640) for 30 minutes the clone 16 cells and uninfected control cells were exposed to 0.5 mCi ^{35}S cysteine (New England Nuclear Research Products, Boston, USA) in 3 ml of freshly added cysteine-free medium for six hours, or to 1.0 mCi of D(6-^{3}H)-glucosamine (New England Nuclear Research Products) in glucose-free RPMI 1640 medium for 12 hours. Labeling of the HUT-78 cells was performed in a similar way, with a labeling period of 12 hours. After their metabolic labeling the cells were washed three times in ice-cold PBS, and a soluble cellular lysate was produced by disruption of the cells with 1 ml of RIPA buffer (25) (0.14 M NaCI, 0.001 M dithiothreitol, 0.01 M Tris HCl pH 8.0, 0.035 % phenyl-methyl-sulfonyl fluoride, and 0.5 % NP 40) followed by centrifugation for 15 minutes at 15,000 g.

Aliquots of 10-30 µl from the above supernatant (corresponding to 10^{5} cpm) were incubated overnight with 4 µl serum samples. Immune complexes if formed were left overnight to bind in the cold to 6 µg of protein A-Sepharose® (Pharmacia AB, Uppsala, Sweden), then carefully washed (three times in 0.5 M NaCl, 0.001 M EDTA, 0.02 M Tris HCl pH 7.6, 1 % sodium deoxycholate and 30 % sucrose, and once in 0.01 M Tris HCl pH 7.6), dried, resuspended in sample buffer (3 % SDS, 1 M urea and 3 % alpha-mercaptoethanol in 1 M Tris-H_3PO_4 pH 6.8), and boiled for three minutes. The samples were analyzed by PAGE on 9 to 16 % polyacrylamide gradient gels with a 2.25 % stacking gel. The gels were

fixed for 1 hour in a solution containing 30 % methanol and 10 % acetic acid, soaked in Amplify (Amersham, Buckinghamshire, England) for 20 minutes, dried and exposed to film (XAR-50, Kodak) for 3-7 days. A ^{14}C labeled protein mixture (phosphorylase b 97,000, BSA 69,000, ovalbumin 46,000, carbonic anhydrase 30,000, lactoglobulin A 18,500) was run parallelly on each gel as the molecular weight reference.

Results

Six virus-specific proteins could be distinctly identified with the aid of HTLV-IIIB-positive sera. These were gp60, gp120, pr55, gp41, p24, p19. As regards pr55, this protein occurred in variable amounts in different batches so it was not subjected to further study. None of the proteins could be detected by immunoprecipitation of uninfected cell lysates or by sera not containing HIV-specific antibodies. Antibody-positive control sera with reactivity to several HTLV-IIIB encoded products precipitated the corresponding proteins from virus-infected cells of both clone 16 and HUT-78. The number of bands was the same for each of the two cell types, but the best quality of the precipitates was obtained with clone 16 because cellular proteins from this clone were labeled to a very minor extent.

An explanation of this phenomenon may be that infection of HUT-78 gives a continuously virus producing cell line, while the virus is lethal for clone 16 cells. With ^{3}H-glucosamine labeled antigen two proteins having molecular weights of 160 Kd and 120 Kd could be precipitated. In antigen labeled with this substance the presence of gp41 could not be demonstrated by means of positive sera; but on the other hand such demonstration was readily feasible with the aid of monoclonal antibody directed against gp41 (gift from R C Gallo). The identities of p19, p24 and their precursors (pr55 and gp41 for p24) were also checked by using monoclonal antibodies.

In addition to the six proteins discussed above, some of the patient sera precipitated proteins of 23 Kd and 27 Kd, these being the products of the sor and orf genes.

DISCUSSION

Example 1: Our results show that cells of human monocytoid origin can be infected with HTLV-IIIB and long term producer cell lines can be established. The T4 antigen seems to be crucial for enabling HTLV-IIIB to bind to the surface of monocytoid cells, since infection of one of the subclones (clone 16) can be blocked by preincubating the cells with anti-T4 antibodies. Interestingly, the T cell leukemia cell line H9 requires a higher concentration of anti-T4 antibodies for blocking the infection than does the monocytoid cell line. The results indicate a close similarity between the HTLV-IIIB receptors on lymphoid and monocytoid cells eventhough receptor density may be lower in the monocytoid cell line than in the T cell line. Our results also show that the higher the precentage of T4-positive cells in a cell culture, the higher will be the susceptibility to HTLV-IIIB infection. Susceptibility is reflected by short latency periods before virus production has got started, and by the strength of the cytopathic effect in the cell culture. In full conformity with the low T4 expression in the parental line (U-937) and in clone 4 it took two months to establish a productive infection or, alternatively, a massive virus inoculum had to be used. None of these cultures showed any cytopathic effect at any time. Clone 16 on the other hand was so sensitive to lysis that no virus producing line could be established in spite of refeeding the culture with fresh uninfected cells. Interestingly, clones 1, 2 and 3 are similar in T4 expression (50-60 % in the cases of 1 and 3; 60-70 % in the case of 2), yet the extent of lytic effect and virus replication is most pronounced in clone 2. Since the subclones of U-937 appear to represent cells frozen at

different levels of monocyte differentiation they may also differ in their ability to modulate T4 expression after viral infection.

Example 2: It is inferrable from the results obtained that the cell lines of the present invention may offer major advantages for the production of viral antigens which are useful for detecting HIV-positive sera.

Table 1

Selected markers for U-937 and for five of its subclones.

Cells	HLA-DR*	T4*	Fc-receptors**	Enzyme activities***
Parental	+	<10 %	35 %	+
Clone 1	++	50-60 %	100 %	+++
Clone 2	++	60-70 %	100 %	+++
Clone 3	+	50-60 %	50 %	+
Clone 4	+	<10 %	50 %	+
Clone 16	+	>95 %	15 %	+

* The HLA-DR data have been obtained using BD-anti-DR FITC in FACS. ++ = all cells highly positive. + = a few positive cells of the same intensity as the average ++ cells but the majority being negative. Figures for T4 are expressed as % positive cells in membrane fluorescence using anti-OKT4 antibodies.

** Fc receptors for IgG are expressed as % of cells making strong and distinct rosettes with IgG-coated SRBC.

*** Esterase (NASDAE), collagenase, elastase, lysozyme. The intensity of histochemical staining was scored in the light microscope as -, +, ++ and +++.

Table 2

Infection of U-937 cells and five of its subclones with HTLV-IIIB.

Cell line		Virus	Days after infection											
			CPE*	RT**	CPE	RT	CPE	RT	CPE	RT	CPE	RT	CPE	RT	CPE	RT
		$x10^3$ cpm	6		10		13		20		28		48		65	
U-937	parental	150	-	0.5	-	0.7	-	0.4	-	0.3	-	0.9	-	3.5	-	79.0
	clone 1	350	-	1.3	-	0.8	-	1.7	+	35.3	++	2.5	-	64.5	-	284.0
	clone 2	150	±	2.1	++	223.5	+++	89.0	+++	64.0	±	19.5	-	215.0	-	87.0
	clone 3	150	-	2.2	-	2.1	-	2.1	-	4.8	+	5.4	-	139.3	-	30.8
	clone 4	150	-	0.4	-	1.4	-	0.3	-	0.2	-	0.3	-	0.6	-	0.1
	clone 4	1800	-	6.5	-	3.6	-	2.9	-	11.9	-	18.9	-	5.9	-	170.4
	clone 16	350	+	14.0	+++	729.6	+++	0.6	deadclone							

* CPE: cytopathic effect. ± <1 %; + 1-5 %; ++ >5 % syncythia formation, fragmentation and cell death; +++ extensive syncythia formation and cell death.

** RT: reverse transcriptase activity, counts per minute 10^3, positive values are represented by 10 times background, underlined.

Table 3

Blocking of HTLV-IIIB replication by anti-T4 antibody.

Cell line	anti-T4 /ug	Dose of virus, dilution	Days after inoculation until signs of virus infection appear 8	11	15	32
H9	0	1:100	0.2**	1.06	0.4	64.2
	2.5	1:100	0.3	0.4	0.3	10.9
	12.5	1:100	0.3	2.3	0.8	0.6
U-937 clone 16	0	undil.	14.0	729.6	CPE***	
		1:10	15.0	CPE		
		1:100	0.4	2.8	5.8	
	2.5	1:10	0.3	1.8	0.6	
		1:100	0.5	1.8	0.5	
	12.5	undil.	0.7	75.1	113.8	
		1:10	0.3	1.4	0.3	
		1:100	0.6	2.4	0.4	

* undiluted = 4×10^5 cpm/ml

** Reverse transcriptase activity x 10^3 cpm. Values 10 times over background were considered positive (underlined)

*** cell culture destroyed by cytopathogenic effects

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PATENT PUBLICATIONS

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Centre National de la Recherihe Scientific (C.N.R.S.).
Inventor: Montagnier, Luc et al.

33. WO-A-8504897
The United States Department of commerce. Inventor:
Gallo, R.C. et al.

APPENDIX

References added in connection with the foreign filings.

34. NICHOLSON, J.K.A. et al. (1986)
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35. EP-A-116949
Eisai Co, Ltd.

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International Application No: PCT/ SE87 / 00628

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 3, line 25 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

European Collection of Animal Cell Cultures (EACCC)
CAMR

Address of depositary institution (including postal code and country) [4]

Porton Down, Salisbury, Wiltshire
United Kingdom SP4 0JG

Date of deposit [5]	Accession Number [6]
1987-10-08	87100805, 87100806, 87100807

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer) Inger Willén

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

CLAIMS

1. Cell line of monocytoid cells capable of unlimited growth in vitro, characterized by being a subclone of a monocytoid wild type.

2. Cell line according to claim 1, characterized by having a T4 content of more than 5 %.

3. Cell line according to claim 1 or 2, characterized in that the wild type is U-937.

4. Cell line according to any of claims 1-3, characterized in that the T4 content exceeds 50 %.

5. Cell line according to any of claims 1-4, characterized in that the cell line can be infected with virus, especially HIV.

6. Cell line according to claim 5, characterized in that the infection is conducive to a productive infection.

7. In vitro method for infecting cell lines capable of unlimited growth for the production of virus particles and fragments thereof, characterized in that cells of a subclone of a monocytoid wild type are infected with virus, said subclone having a stable T4 content, preferably exceeding about 50 % and its cells being capable of giving a productive infection.

8. Method according to claim 7, characterized in that the subclone infected is a subclone of U-937.

9. Method according to either of claims 7 or 8, characterized in that HIV is employed for the infection.

10. Method in producing cell lines capable of unlimited growth in vitro and of being infected by virus, especially by HIV, characterized by cloning in a manner known per se a wild type of a monocytic cell line, followed by selecting and recovering proliferating clones which have a T4 content exceeding 5 %, preferably exceeding 50 %.

11. Method according to claim 9, characterized by cloning U-937.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE87/00628

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC 4

C 12 N 5/00, 7/00, A 61 K 39/21, G 01 N 33/569, C 12 Q 1/70

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 3	A 61 K 39/21; C 12 N 5/00, /02, 7/00, /02; C 12 Q 1/70; G 01 N 33/569, 33/571
US Cl	435:5, 235, 239, 240

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
E	WO, A1, 87/06258 (THE UNITED STATES DEPARTMENT OF COMMERCE) 22 October 1987	1-16
E	EP, A2, 0 220 970 (INSTITUT PASTEUR) 6 May 1987	1-11
E	WO, A1, 87/04457 (INSTITUT PASTEUR) 30 July 1987	1-11
Y	Journal of Immunology, vol. 137, No. 1, July 1, 1986, p. 323-29 (NICHOLSON JANET K. A. et al) "IN VITRO INFECTION OF HUMAN MONOCYTES WITH HUMAN T LYMPHOTROPIC VIRUS TYPE III/LYMPHADENOPATHY-ASSOCIATED VIRUS (HTLV-III/LAV)". See page 323, col. 2, second paragraph, page 327, col. 2 and page 328, col. 1, lines 16-17 from below.	1-3, 5, 6, 10
Y	Virology, vol. 147, 1985, pages 441-448 (LEVY J. A. et al) "AIDS-Associated Retrovirus (ARV) Can Productively Infect Other Cells Besides Human T Helper Cells". See page 444, col. 1, lines 3-5 and second paragraph.	1-3, 5, 6, 10

.../...

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published 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; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1988-03-14	1988 -03- 24
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Carl Olof Gustafsson

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

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature, Vol. 312 20/27 December 1984, pages 763-767 "The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus". (DALGLEISH A. G. et al) See in particular tabel 2	1-11
A	J. Clinical Invest. Vol. 77, May 1986, pages 1712-15 "Infection of Monocyte/Macrophages by Human T Lymphotropic Virus Type III". (HO D. D. et al)	1, 5, 6
A	Science, Vol. 229, 9 August 1985, pages 563-66 "Infection of HTLV-III/LAV in HTLV-I-Carrying Cells MT-2 and MT-4 and Application in a Plaque Assay". (HARADA S. et al) See page 564, col. 1 and col. 3, second paragraph	1, 5, 6
A	WO, A1, 85/04897 (THE UNITED STATES DEPARTMENT OF COMMERCE) 7 November 1985	1, 5, 6