

## DIFFERENTIATION AND TRANSFORMATION OF HEMATOPOIETIC CELLS IN CULTURE

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Recent findings of viral specific RNA in human tumors such as human breast cancer (1), leukemias (2), sarcomas (3) and lymphomas (4) which are related to RNA of RNA tumor viruses of animals support the idea of a virus aetiology in human cancer. The establishing of cell cultures of human origin transformed in vitro should provide a powerful aid in the search for human cancer. Diseases which are related to leukemia and which can be considered as being preleukemic stages seem to be ideal systems to study the malignant transformation in man. In addition the disease to be studied should be reasonably frequent and the disease entity well characterized. Furthermore, during the course of the disease some similarities should occur between the disease and experimental RNA tumor virus induced diseases in animals. This seems to be true for Polycythaemia vera (PV).

PV is a proliferative disease of the hematopoietic system, mainly of the erythropoietic compartment. Often PV changes, especially after irradiation and chemotherapeutical treatment, to acute leukemia, chronic myelogenous leukemia or myeloid metaplasia (5). In the development of the Friend virus induced leukemia in mice a similar polycythemic stage is observed (6). In the Friend polycythemia as well as in PV, an unregulated multiplication, but normal differentiation of the cells of the red compartment is observed. In both diseases the abnormal erythropoietic precursor cells do not respond to the proteohormone erythropoietin. We have shown that Friend virus transformed erythropoietic tissue culture cells also do not respond to erythropoietin (7). For these reasons bone marrow cells of patients with Polycythaemia vera seem to be an appropriate material for establishing cultures to study processes leading to leukemic transformation in man.

## Cell Culture:

Bone marrow of a 69-year old male patient with PV has been cultivated. After a proliferative phase of two weeks the cells successively became fibroblastoid and formed monolayers. After 8 weeks the contact inhibited monolayer cultures did not contain cells in suspension. During the ninth week a "piling up" has been observed on different loci of the monolayers of two parallel cultures. The spontaneously transformed cells grow in suspension as single cells or in clusters. They have a generation time of about 24 hours. Figure 1 shows the possible correlation between the time course of the cell culture and the clinical course of the disease.

Cytologically there was no difference between the cell types of the culture and normal bone marrow during the first 3 weeks. After the transformation, however, 90 % of the cells are blasts, 8 % orthochromatic erythroblasts and a small portion lymphoid cells (see Table 1).

## Globin synthesis

The cytological observations of orthochromatic erythroblasts led to biochemical investigations for globin synthesis, although the cells gave a benzidine negative reaction. Cells were labelled with  $^3\text{H}$ - and  $^{14}\text{C}$ -leucine and globin chains were isolated (8, 9). Radioactively labelled tissue culture protein eluted as defined peaks together with the  $\alpha$  and  $\beta$  carrier globin. Tryptic fingerprint analysis of  $\alpha$ - and  $\beta$ -chains were carried out as described (10). Preliminary results show that  $\alpha$ - and

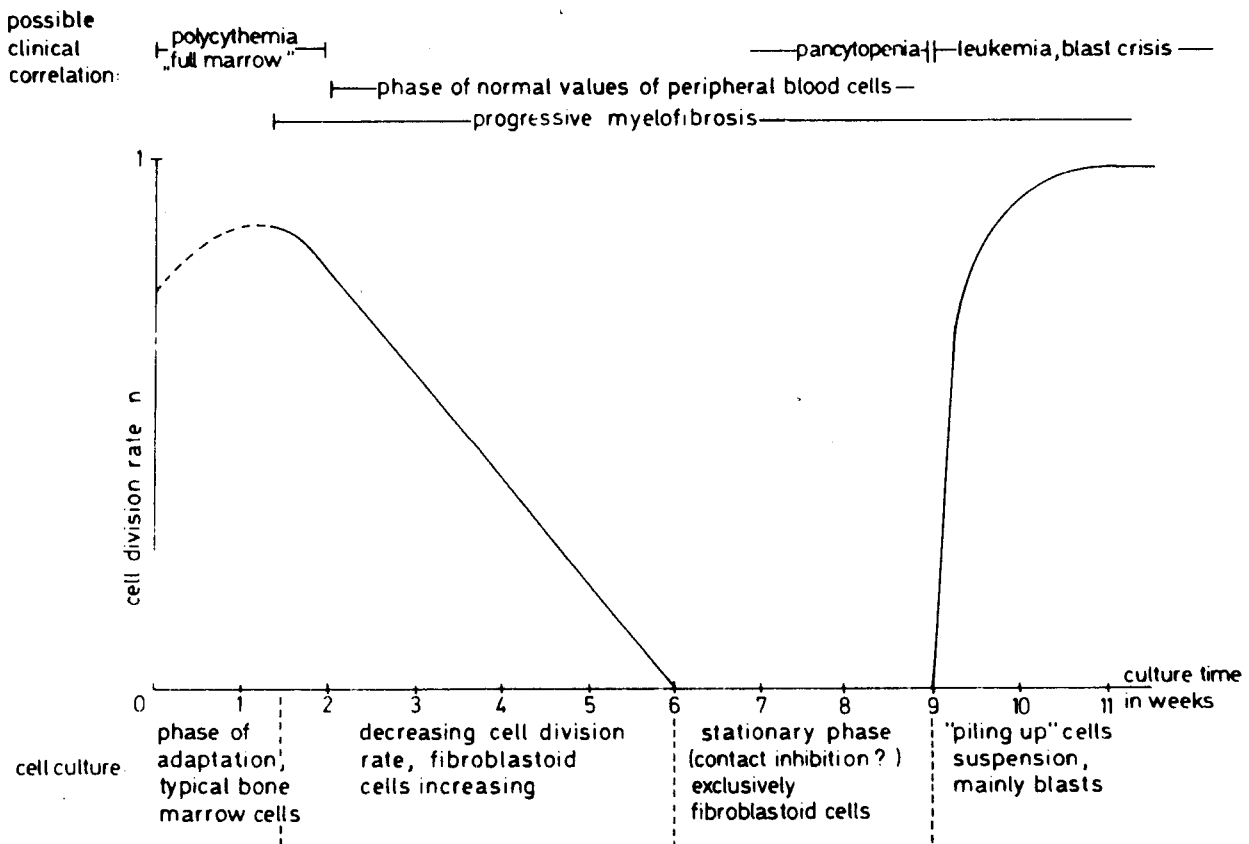


Fig. 1: Schematic representation of changes leading to PV tissue culture and possible clinical correlation.

**Table 1: Cytological and cytochemical classification of the cells during the process of establishing the cell culture**

culture time in weeks	granulopoiesis			poly chromatic	erythropoiesis		
	segm	eos	precursor cells		ortho	ery	monocytes
3	+	+++	+++	++	++	-	+
9	+	-	-	-	+	+	-
12	-	-	-	-	+	-	-
16	-	-	-	-	+	-	-
22	-	-	-	-	++	-	-

	lymphocytes	blasts	lymphoid cells
3	+	-	-
9	-(+)	(+)	+
12	-	+++	++
16	-	++++	+
22	-	++++	(+)

$\beta$ -type peptides are present. Stimulation with 1 % DMSO for 5 days had no effect on globin synthesis contrary to the Friend virus induced erythroleukemic mouse cells in culture (10).

### Karotype

The karyotype is hyperdiploid. All cells (n = 20) contained 48 chromosomes. The 2 additional chromosomes appear as one large metacentric and one large submetacentric chromosome (see Figures 2 and 3). Furthermore, a change in one chromosome of the F-group (deletion or inversion) can be observed. This anomaly is considered to be a specific aberration for some cells in several patients with PV (11).

### High molecular weight RNA and reverse transcriptase

The possible role of the RNA dependent DNA polymerase as a key enzyme in neoplastic transformation of human cells has guided several investigations since the discovery of the enzyme in RNA tumor viruses (12, 13). For the simultaneous detection of high molecular weight RNA and reverse transcriptase in PV cells the method of SCHLOM and SPIEGELMAN (14) was applied. It was found earlier that the initial DNA product of the reaction of reverse transcriptase on 70S RNA is complexed via hydrogen bonds to the template. This was demonstrated by the unusual position of DNA product in glycerol velocity and  $\text{Cs}_2\text{SO}_4$  equilibrium gradients. After mild heat denaturation the DNA product was detected in the expected positions in  $\text{Cs}_2\text{SO}_4$  and glycerol gradients (15, 16).

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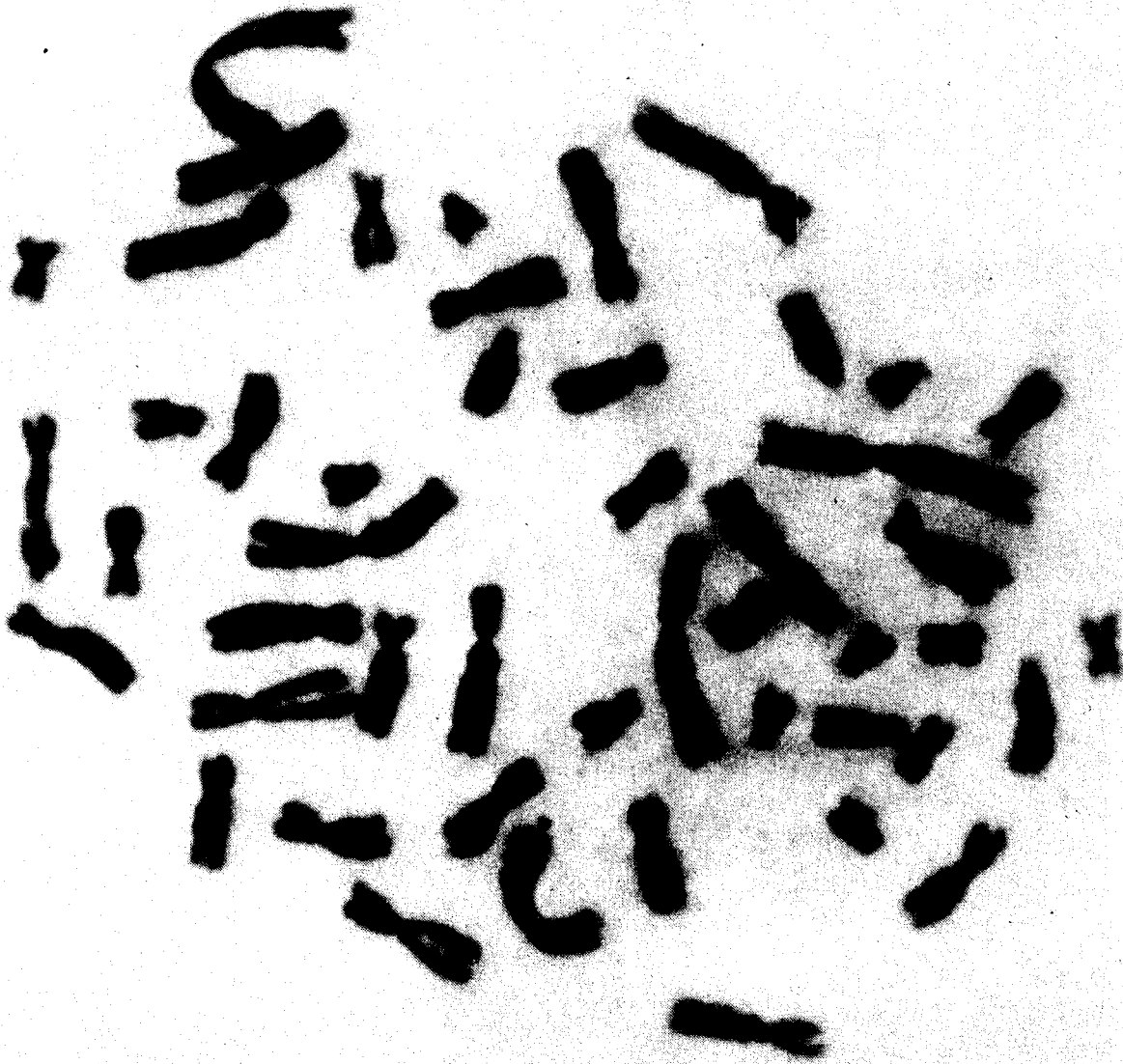


Fig. 2: Metaphase chromosomes of a PV cell

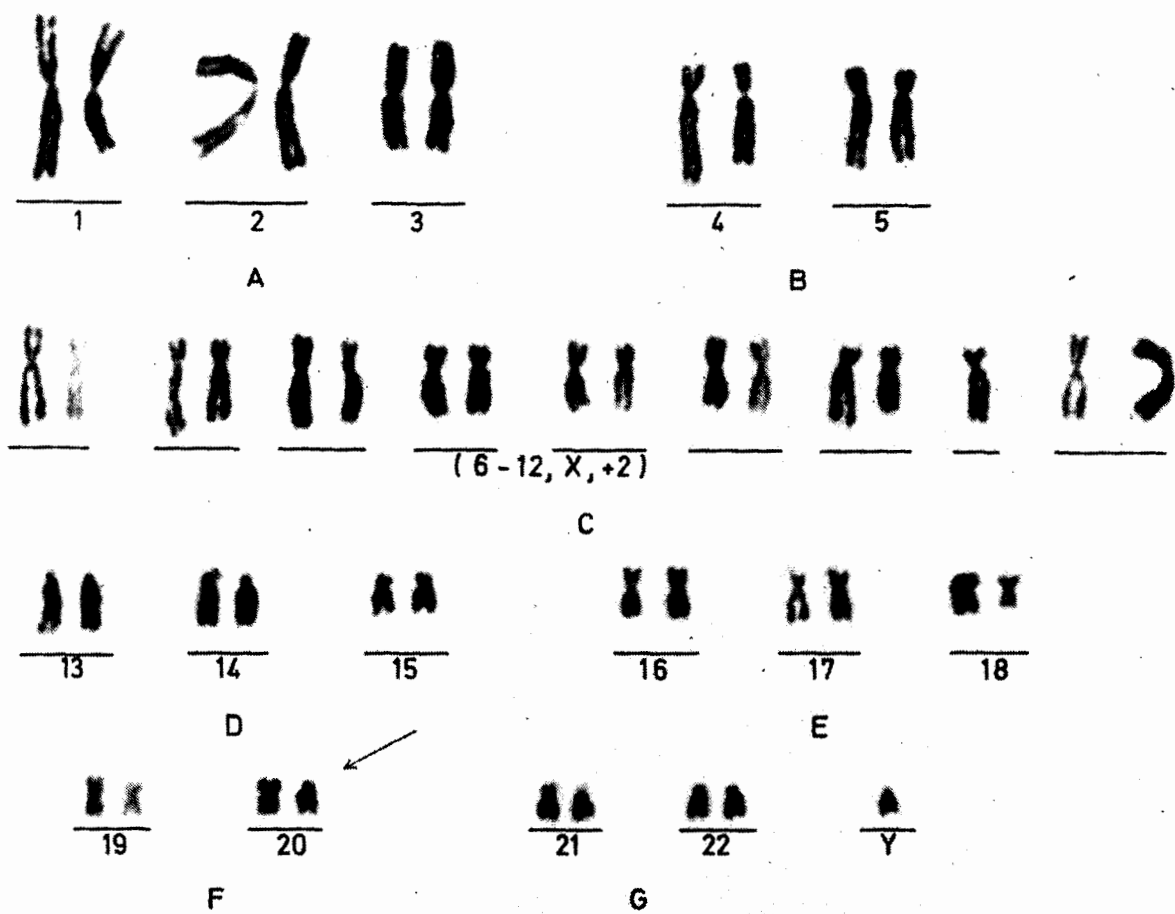


Fig. 3: Idiogram of the chromosomes shown in fig. 2, Arrow indicates the changed F-group chromosome

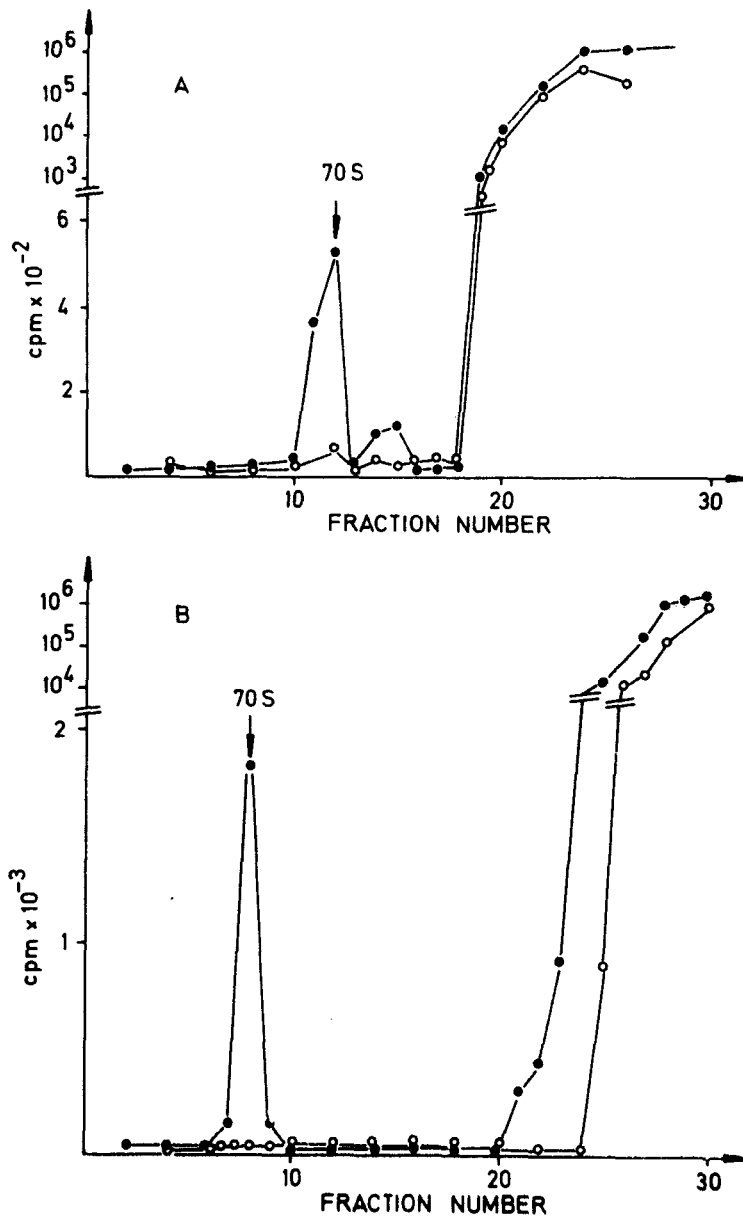


Fig. 4: Detection of high molecular weight <sup>3</sup>H-DNA/RNA complex in PV cells. Material with a density of about 1.16–1.19 g/cm<sup>3</sup> was collected from a 20–60 % sucrose gradient and pelleted by high speed centrifugation. The pellet was incubated in 0.5 ml of 0.3 % Nonidet P40, 0.005 M DTT, 0.01 M Tris HCl, pH 8.3 for 15 min. at 0 °C. DNA was synthesized in a RNA dependent DNA synthesizing mixture (1 ml) containing 0.01 M Tris-HCl, pH 8.3, 0.02 M NaCl, 0.005 M MgCl<sub>2</sub>, 5 × 10<sup>-4</sup> M of each dATP, dCTP, dGTP and 5 × 10<sup>-5</sup> M <sup>3</sup>H-dTTP (3500 cpm/pmol). 50 μg/ml actinomycin D was added to inhibit DNA dependent DNA synthesis. After synthesis for 15 min. at 37 °C the reaction was stopped by addition of 1 % SDS and 0.2 M NaCl final concentration. The mixture was deproteinized by phenol-cresol extraction. The aqueous phase was layered on a 15–30 % linear glycerol gradient and centrifuged in a Spinco SW 40 rotor at 38,000 rpm for 3 hours at 4 °C. Fractions were collected through the bottom and assayed for radioactivity. A: one aliquot was preincubated with RNase (20 μg/ml) for 30 min. at 37 °C (o), the other aliquot was directly applied to the gradient (·). B: reaction mixture contained <sup>3</sup>H-dCTP and <sup>3</sup>H-dGTP, (o) preincubation with RNase, (·) no RNase.

A high speed pellet fraction was isolated from the cytoplasm of PV cells, resuspended and centrifuged through a 20–60% linear sucrose gradient. Single fractions of the gradient have been tested for incorporation of  $^3\text{H}$ -TMP on the synthetic template poly rA/oligo dT. Material of a density of 1.16–1.19 g/ml was used for the simultaneous detection of high molecular weight RNA and reverse transcriptase and for the isolation of the enzyme. After incubation of the material for 15 min. at 0 °C in a buffer containing 0.3% of the detergent Nonidet P40 a reverse transcriptase reaction was carried out in the presence of actinomycin D to

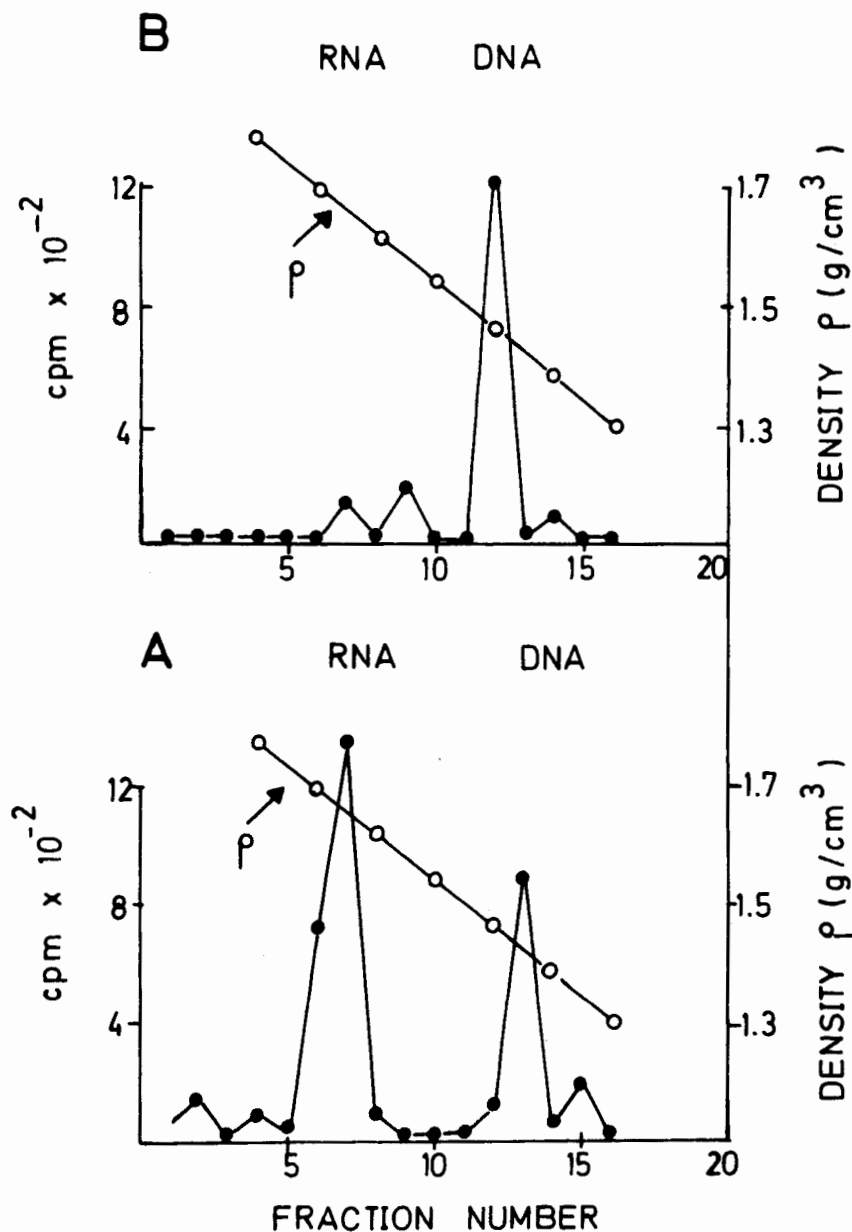


Fig. 5:  $\text{Cs}_2\text{SO}_4$  equilibrium density centrifugation of the  $^3\text{H}$ -DNA product. Appropriate fractions from the glycerol gradient were pooled and the radioactive material was precipitated with ethanol in the presence of yeast RNA carrier. The precipitate was dissolved in 11 ml half-saturated caesium sulphate ( $\rho = 1.51$  g/ml) and centrifuged in a Spinco 75 Ti rotor at 44,000 rpm for 48 hours at 20 °C (Fig. 5a). The radioactive material, which bands at a density of 1.62–1.66 g/ml, was subjected to alkaline hydrolysis (0.4 N NaOH, 24 hours, 43 °C), neutralized with HCl and re-centrifuged in  $\text{Cs}_2\text{SO}_4$  under the same conditions as described above (Fig. 5b).

inhibit DNA dependent DNA synthesis. The reaction was stopped by SDS treatment, deproteinized with phenol-cresol and centrifuged in a 15–30 % glycerol gradient with a size marker (Figure 4). The radioactive moiety of the 70S region was subjected to Cs<sub>2</sub>SO<sub>4</sub> equilibrium density centrifugation (Fig. 5). Most of the radioactivity is found in the RNA region (1.64–1.68 g/ml), although some material is found in the DNA region, probably due to a breakdown during the isolation procedure (Fig. 5a). After alkaline hydrolysis the same material bands in the DNA region (Fig. 5b).

After ultrasonication of the high speed pellet the DNA polymerase activity was extracted with a buffer containing a nonionic detergent and purified on DEAE- and phosphocellulose columns. The activity which elutes from the phosphocellulose column represents a 220-fold purification. Table 2 shows the response of the human PV DNA polymerase to various templates.

The complete reaction mixture consisted of the following in 0.05 ml: 10 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM manganese acetate, 20 µg/ml actionmycin D, 0.5 mM of each dATP, dCTP, dGTP, 0.1 mM <sup>3</sup>H-dTTP (35 cpm/pmol), 0.5–0.8 µg of PV DNA polymerase, 1 µg(dT)<sub>12–18</sub>, 2 µg 9–12 S globin mRNA, 1 µg globin mRNA, (purified by gel electrophoresis), 2 µg R17 RNA. Reactions were carried out at 37 °C for 30 min.

Table 2. Template response of PV DNA polymerase

Template	pmol <sup>3</sup> H-dTTP incorporated in 30 min. at 37 °C	pmol <sup>3</sup> H-dCTP incorporated in 30 min. at 37 °C
poly rA (dT) <sub>10</sub>	72	—
p-12S Globin mRNA (purified by sucrose gradient centrifugation)	—	3.8
Globin mRNA (purified by gel electrophoresis)	51	10.4
R 17 RNA	0.2	0.2

### Electronmicroscopy

Electronmicroscopic studies on cells and homogenates reveal particles with diameters ranging from 900 to 1200 Å. By the study of numerous cells we found these particles in clusters with an average of 8 particles per cluster. The investigation did not include the examination of serial sections, so that a true statistical analysis was not possible. Figure 6 shows a section through a cell with a group of particles. In Fig. 7 a single isolated particle with a diameter of 1200 Å is shown. The electron-dense core and triple-layered structure of the envelope are discernible.



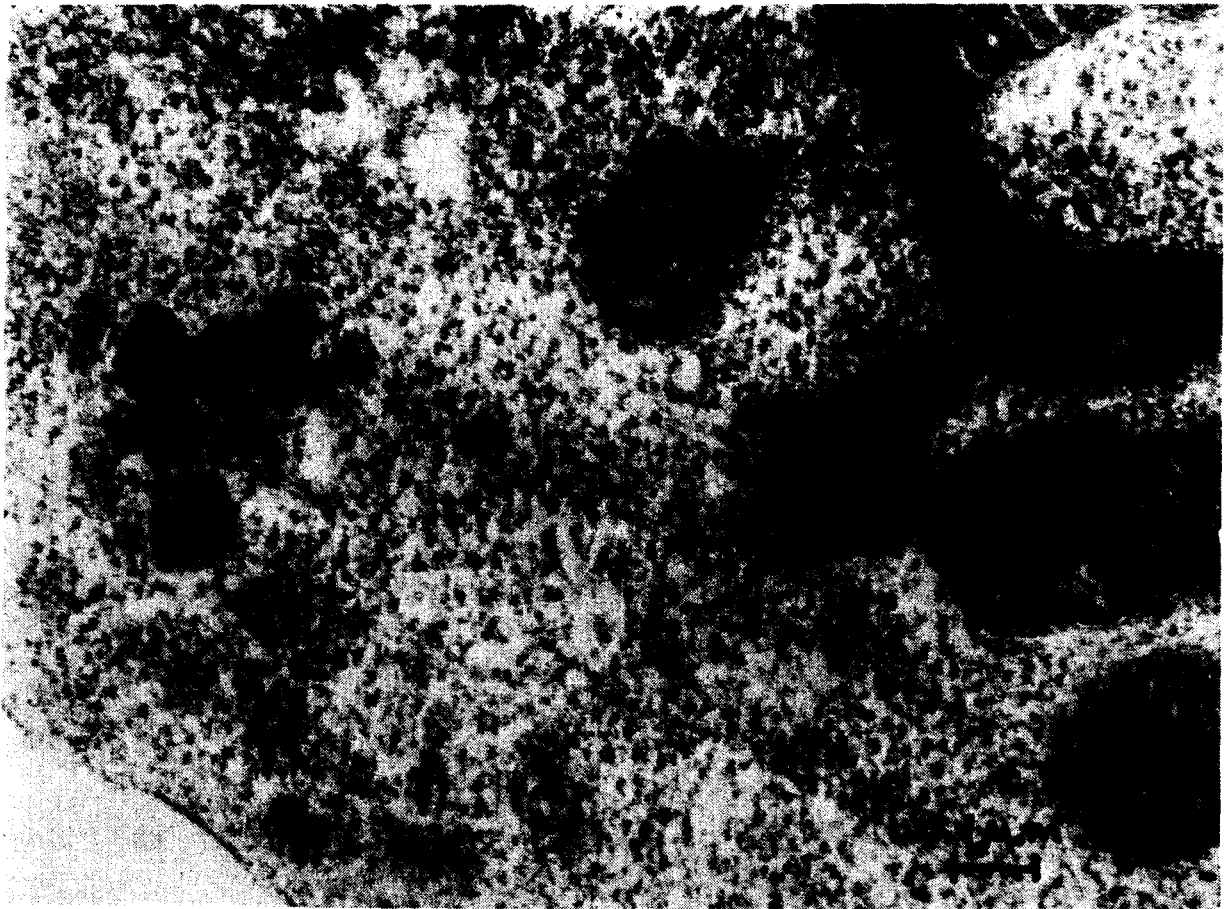


Fig. 6: Ultrathin section of a PV-cell. The cell was fixed with 2.5 % glutaraldehyde in 0.1 M potassium-sodium-phosphate-buffer, pH 7.2, followed by postfixation with 1 % osmiumtetroxide in isotonic sodium-veronal-acetate buffer, pH 7.2 and with 0.5 % uranylacetate in 0.1 M acetate buffer, pH 3.9. The specimens were dehydrated with ethanol and embedded in epon. Sections were stained with 3 % aqueous uranyl-acetate and 0.3 % lead-citrate. Photographs were taken in a JEOL Jem 100 B electron-microscope at 80 KV. Mag.: 80,000 x

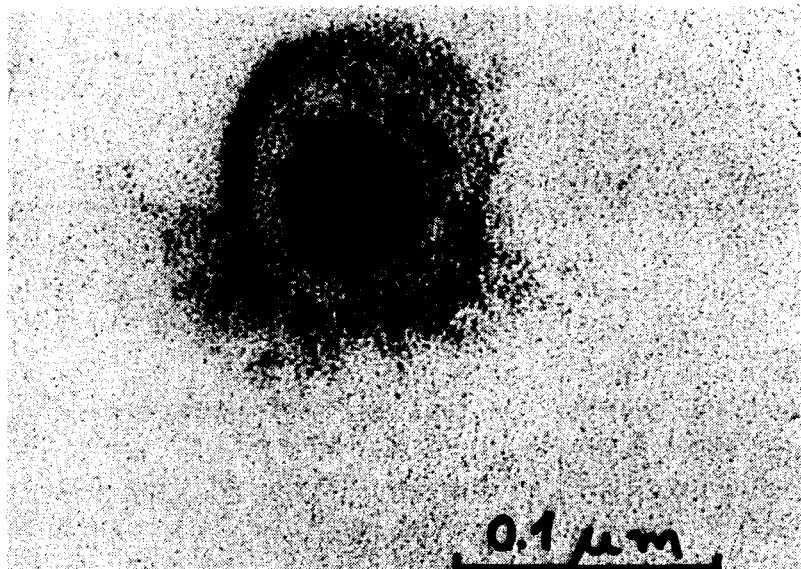


Fig. 7: Ultrathin section through a cytoplasmic pellet. Fixation and embedding as in Fig. 6. Mag.: 250,000 x

## Summary

We have established a cell culture line from bone marrow of a patient with a Polycythaemia vera (PV) disease. During the time course of the establishing of the culture we observed a spontaneous transformation in vitro. Cytologically no difference was found between cell types of the culture and normal bone marrow during the first 3 weeks. After the transformation, however, 90 % of the cells are blasts, 8 % orthochromatic erythroblasts and a small portion lymphoid cells. The karyotype of the cells in culture is hyperdiploid and contains 48 chromosomes. Furthermore, a change in one chromosome of the F-group (inversion or deletion) is observed. The cells produce adult human globin and contain particles with high molecular weight RNA and reverse transcriptase activity. Investigations by electron microscopy revealed virus like particles.

## Acknowledgments

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (G. G., J. K., N. K., W. O.).

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