Protein parameters of differential gene activation during development and tumorigenesis

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ABSTRACT Various models of normal and abnormal developmental systems were addressed to get an insight into molecular parameters of cell differentiation at the level of protein gene products. Electrophoretic analysis of heterogeneous protein mixtures permitted qualitative analysis of developing systems, particularly during organogenesis in mammals, as well as of neoplastic growth in the animal and plant kingdoms. From our earlier findings indicating that the definite protein patterns characteristic of adult organs are acquired long after the adult morphological and histological characteristics of these tissues have developed, it has been repeatedly proven that quantitative changes in whole proteins is not a dependable indicator of cell differentiation.

KEY WORDS: *cell differentiation, protein gene products, crown-gall tumor, interferon, human cervical cancer*

Introduction

Molecular analyses of differentiation during organ and tissue development have long attracted attention to protein molecules as the protein gene products which either contribute to easily identifiable structures of the cell, or whose enzymatic action confers to the differentiated cells distinctive metabolic capacity. Proteins are relatively stable. They yield to various extraction and analytical procedures, and indicate the changes in developing cells known as differential gene activation, giving rise to the structural and functional potential of the differentiated tissue (Serman and Skreb, 1970).

Rat organogenesis

Molecular studies on developmental changes during organogenesis in the rat were initiated in an attempt to detect known adult characteristics of different organs and tissues as early as possible during intrauterine and early postnatal development (Serman and Skreb, 1970). Protein patterns obtained by polyacrylamide gel electrophoresis (PAGE) under native conditions from a number of rat organs were studied during final differentiation stages. Soluble proteins were extracted from the rat brain, heart and skeletal muscle during their late fetal (17, 19 and 21 day-old fetuses) and postnatal development (3, 30 and 90 day-old animals). The adult organs showed specificity of their protein patterns as indicated by the relative quantitative proportions and distribution of the separated protein bands. The composition of soluble proteins of morphologically already well defined organs in the late fetal stage of development is not changed by the event of birth and is preserved unmodified until the early neonatal period. Significant developmental changes in protein composition leading to the formation of the adult protein pattern take place only between the 3rd and 30th postnatal day. Therefore, the definite adult protein patterns apparently do not appear in some organs until all the morphological and histological characteristics are acquired (Deuchar, 1973). The descriptions of the changes in soluble proteins in developing organs and tissues therefore show only a small part of all the events of differentiation. Deuchar (1973) considers the most significant finding of this study to be the fact that the definite pattern characteristic of each adult organ was not acquired until after birth, *ie.* long after the adult morphological features of these tissues had developed. This finding, according to Deuchar, discourages one from regarding changes in whole proteins as the best indicators of early differentiation (1973).

Development is a continuous, time-dependent process

Polyacrylamide gel electrophoresis is used more and more in various biomedical applications. Biological and medical studies dealing with molecular inventories at the cellular, subcellular, or supracellular levels, or those dealing with developmental processes provide two different types of information: «signals», or messages

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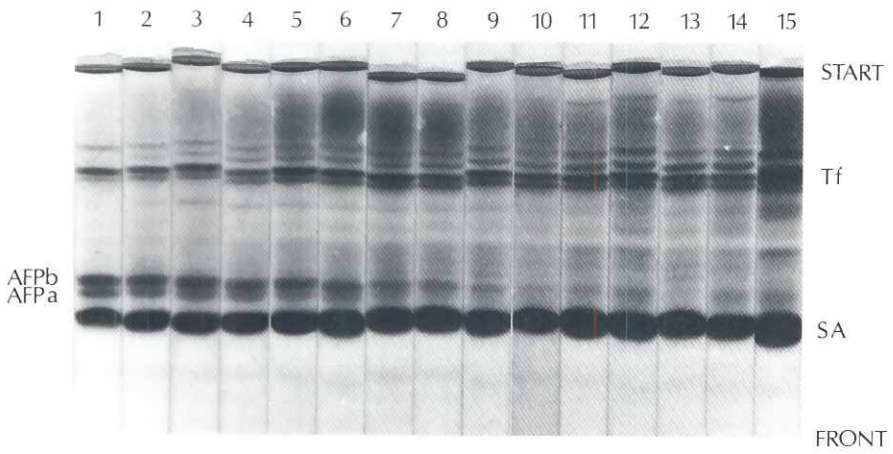

Fig. 1. Alpha-fetoprotein (AFP) changes during postnatal development of the rat as observed by polyacrylamide gel electrophoresis under non-denaturing conditions (PAGE). *Lane numbers followed by days of fetal (f) or post-natal (pn) age.* **(1)** *Serum of the 21 f.* **(2)** *2 pn.* **(3)** *6 pn.* **(4)** *10 pn.* **(5)** *12 pn.* **(6)** *14 pn.* **(7)** *16 pn.* **(8)** *18 pn.* **(9)** *20 pn.* **(10)** *22 pn.* **(11)** *24 pn.* **(12)** *26 pn.* **(13)** *28 pn.* **(14)** *30 pn.* **(15)** *90-day-old rats. SA, serum albumins; AFPa, fast and AFPb, slow fraction; Tf, transferrin.*

on the changing patterns in polyacrylamide gels intended for comparison (Serman and Skreb, 1972).

A continuous or analogous signal describes our insight into changing patterns arising from a continuously changing system like e.g. protein changes during development of the same organ: the brain, liver or in serum proteins. This signal is presented as a family of basic patterns obtained by densitometric recordings, in an attempt to acquire quantitative information on changes of a single parameter: protein composition of the complex system.

A discontinuous signal, on the contrary, describes changes and variations between the compared patterns, eg. protein patterns obtained from different organs at the same developmental stage, and which are therefore devoid of a successive relationship among them. This type of signal arises from comparative biochemical analysis in population genetics, clinical diagnostics or any attempt to screen for mutagenic activity.

Developmental changes in rat alpha-fetoproteins

In the early stage of development of this technique, before the improvements and breakthrough to two-dimensional separations by O'Farrell (1975), we attempted to improve our separation conditions and possibilities of comparing many one-dimensional patterns by establishing the colinearity of protein patterns (Serman and Skreb, 1972). We varied the time scale between the fastest and the slowest gel in order to allow the tracking dye front in all the tubes of a single run to reach the same distance within the separating gel. Protein patterns generally achieved satisfactory colinearity of separated bands, whereby the appearance of new, or the disappearance of old, bands from the pattern, or quantitative changes in the same band at various developmental stages can be easily followed. Fig. 1 shows the disappearance of rat alpha-fetoproteins from the serum protein pattern during postnatal development. This classical oncofetal protein is separated in rat into two discrete electrophoretic fractions: AFPa and AFPb as shown by Wise *et al.* (1963) in starch gel, by Watabe (1974) and Watanabe *et al.* (1975) in non-denaturing polyacrylamide gel electrophoresis (PAGE) and under denaturing condition in the presence of sodium dodecyl sulfate (SDS-PAGE) by Kerckaert and coworkers (1977). We have observed the gradual decrease of both alpha-fetoprotein fractions (Fig. 2) and their quantitative substitution with serum albumins (Serman and Skreb, 1972), as the classical example of continuous «signal». Akira and Nishi (1991) explain the molecular basis for the two electrophoretic variants of rat alpha-fetoproteins: the fast variant, AFPa contains one sugar chain per molecule, while the slow variant, AFPb contains two sugar chains per molecule of rat AFP, having a molecular weight of 67000.

The same technique, when used for following the development of pattern complexity as various organs and their cytosol extracts are compared at horizontal developmental transects, provides

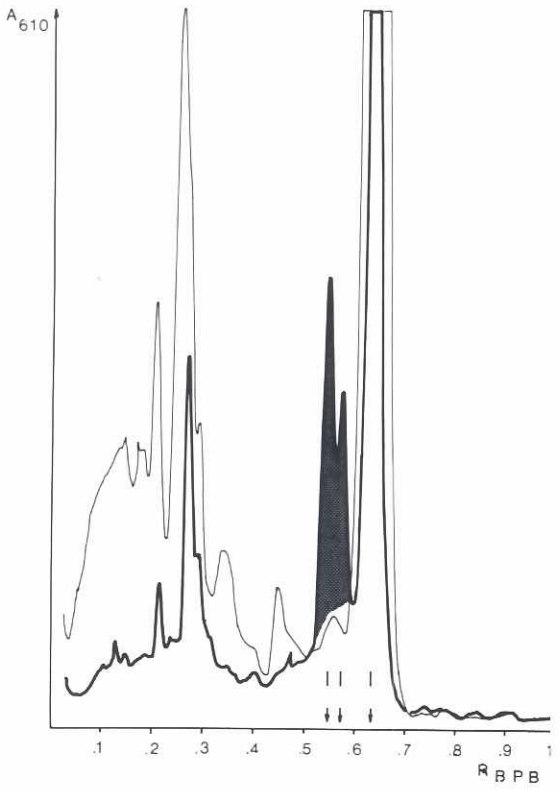

Fig. 2. Comparison of densitograms of rat serum albumins and alpha-fetoproteins between late fetal and adult animal. *Absorbance was measured at 610 nm in the Joyce Loebl Chromoscan, with start at the left side and front of the bromphenol blue (BPB, 1.0) at the right side.*

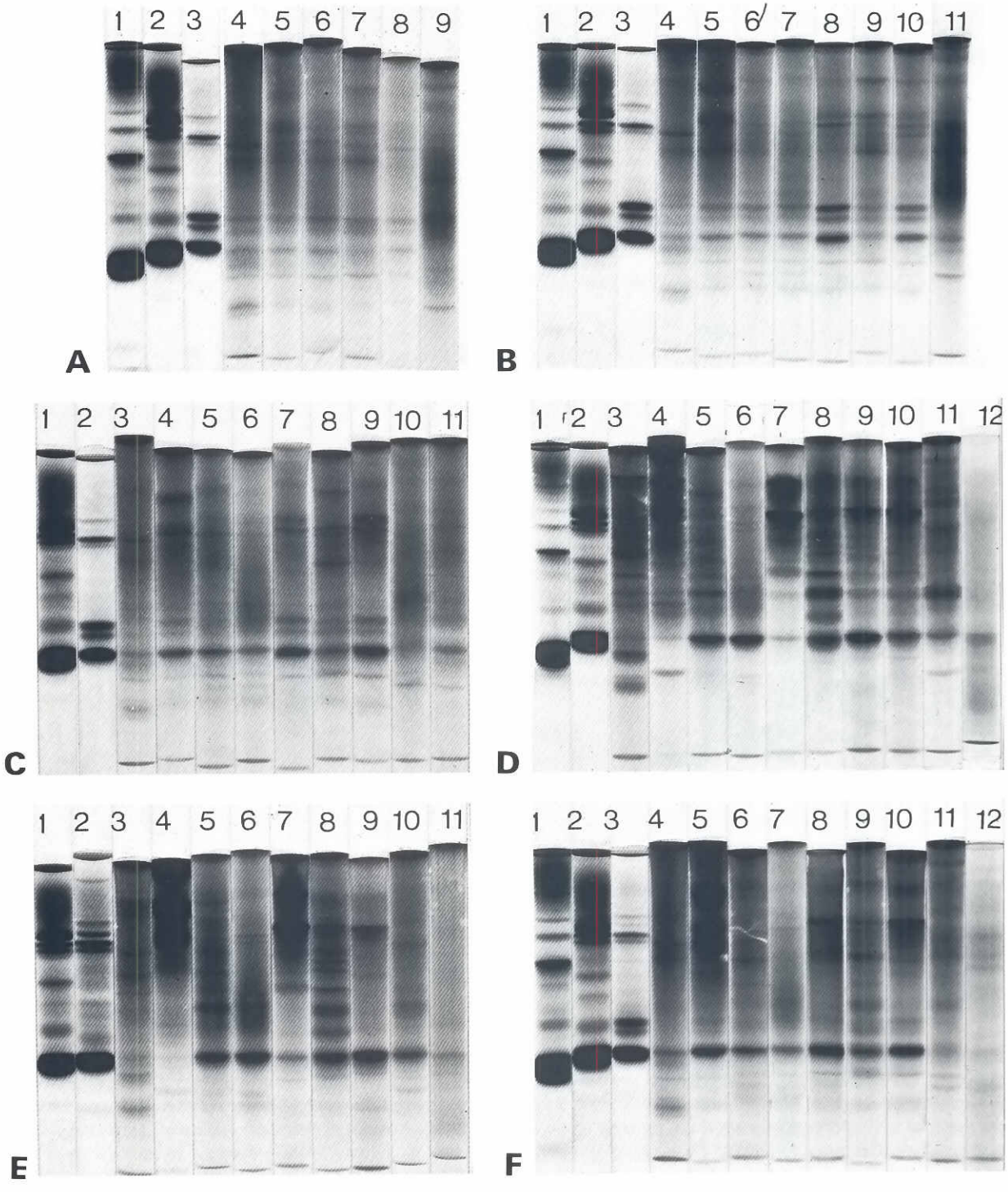

Fig. 3. Differentiation of cytosol proteins in various organs of the rat during perinatal and postnatal development. (A) *17-day-old fetus. Controls in lane 1 - human serum, 2 - adult rat serum, 3 - fetal rat serum. Fetal cytosols in lane 4 - brain, 5 - eye, 6 - lung, 7 - heart, 8 - tongue, and 9 - liver.* **(B)** *19-day-old fetus. Controls in lane 1 - human serum, 2 - adult rat serum, 3 - fetal rat serum. Fetal cytosols in lane 4 - brain, 5 - eye, 6 - kidney, 7 - lung, 8 - muscle, 9 - heart, 10 - tongue, and 11 - liver.* **(C)** *21-day-old fetus. Controls in lane 1 - adult rat serum, 2 - fetal rat serum. Fetal cytosols in lane 3 - brain, 4 - eye, 5 - kidney, 6 - lung, 7 - muscle, 8, heart, 9 - tongue, 10 - liver, and 11 - stomach.* **(D)** *3 days* post partum. *Controls in lane 1 - human serum, 2 - adult rat serum, 3 - fetal rat serum. Newborn rat cytosol in lane 4 - brain, 5 - eye, 6 - kidney, 7 - lung, 8 - muscle, 9 - heart, 10 - tongue, 11 - liver, and 12 - diaphragm.* **(E)** *30-day-old rats. Controls in lane 1 - adult rat serum, 2 - human serum. Young rat cytosol in lane 3 - brain, 4 - eye, 5 - kidney, 6 - lung, 7 - muscle, 8 - heart, 9 - tongue, 10 - liver, and 12 - stomach.* **(F)** *90-day-old rat. Controls in lane 1 - human serum, 2 - adult rat serum. Adult rat cytosol in lane 3 - brain, 4 - eye, 5 - kidney, 6 - lung, 7 - muscle, 8 - heart, 9 - tongue, 10 - diaphragm, 11 - liver, and 12 - stomach.*

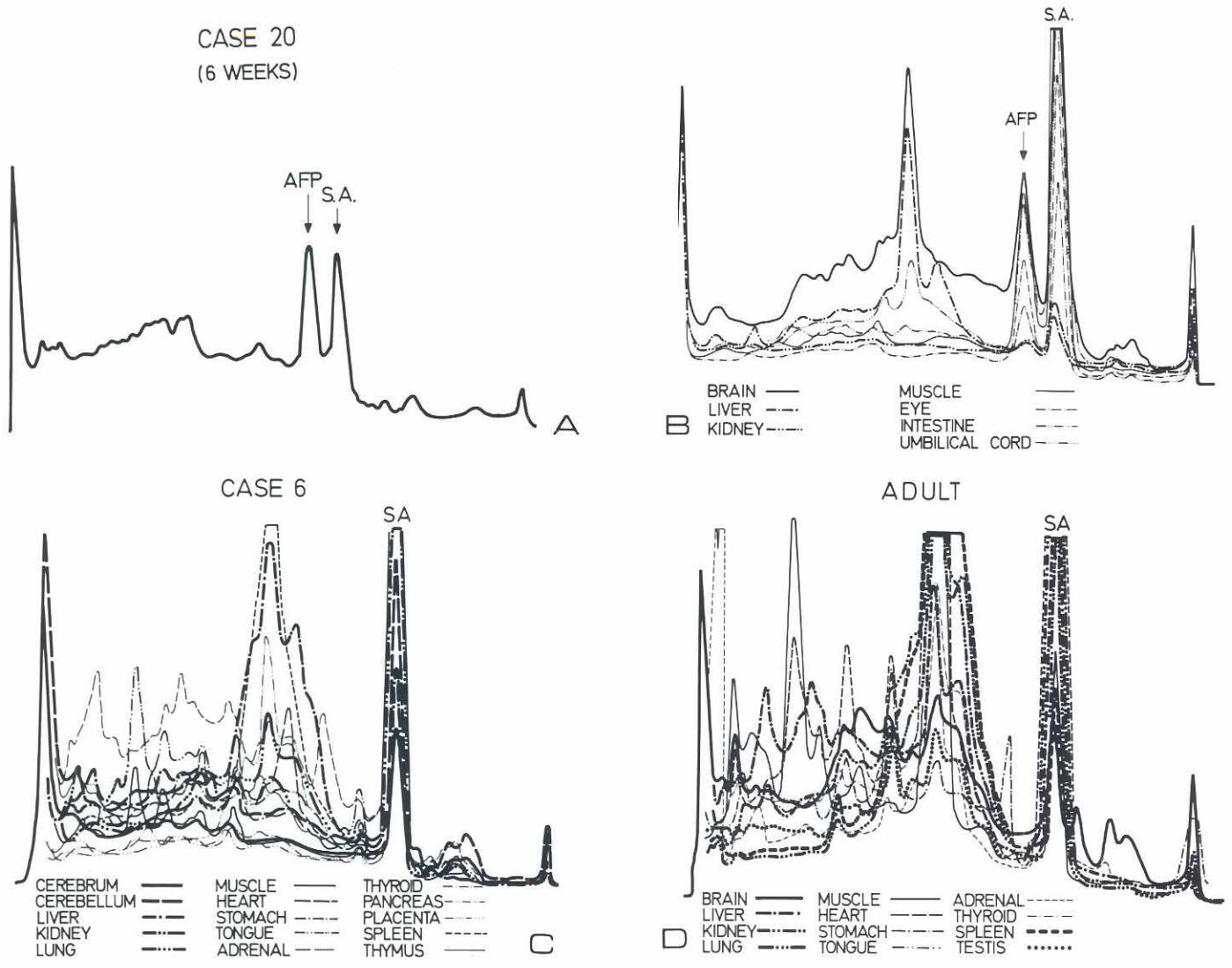

Fig. 4. Densitograms of PAGE patterns of human cytosol proteins extracted from pathological material of various organs from spontaneous abortions and adult man after heart failure. (A) *Embryo of 6 weeks. The cytosol pattern of the whole embryo is dominated by serum albumins (SA) and alpha-fetoproteins (AFP), which in human embryo exist as a single fraction.* **(B)** *Embryo of 8 weeks. Serum albumins and AFP still dominate in cytosols of several organs studied.* **(C)** *Fetus of 19 weeks. Cytosols of various organs present much more diversified organ-specific protein patterns, with serum albumins as major component, and AFP only in traces.* **(D)** *Adult man who died of heart failure. Presents the most differentiated organ-specific patterns of soluble proteins.*

information which might give a visual image of differential gene activation during rat development (Fig. 3). Probably the densitograms of cytosol extracts of various human organs, obtained from pathological material and spontaneous abortion, present an even better picture of differentiation as differential gene activation during development, and acquisition of ever-increasing protein pattern complexity (Fig. 4).

Lactate dehydrogenase isoenzymes during human development

The earlier work of Markert and Ursprung (1962) sets one of the most impressive frames for differential gene expression of enzyme subunits appearing in multiple molecular forms of lactate dehydrogenase. The aim of our work was to analyze the patterns of lactate dehydrogenase (LDH) isoenzymes, and their changes in various organs during the intrauterine development of man, interrupted by spontaneous abortion. LDH-isoenzymes were separated by polyacrylamide gel electrophoresis and identified by histochemical reaction in soluble protein extracts of various organs. This was carried out in a series of about twenty spontaneous abortions, some of which displayed the typical picture of habitual abortion (Serman, 1982).

LDH-isoenzymes are a very convenient model for detection of metabolic disturbances, because they represent the sensitive system of both genetic and epigenetic controls over expression of three structural genes. These controls are furthermore dependent on the oxygenation status of the fetus. Findings for an adult man, 42 years old, who died from heart infarction show results (Fig. 5) which are in agreement with those expected for this cause of death.

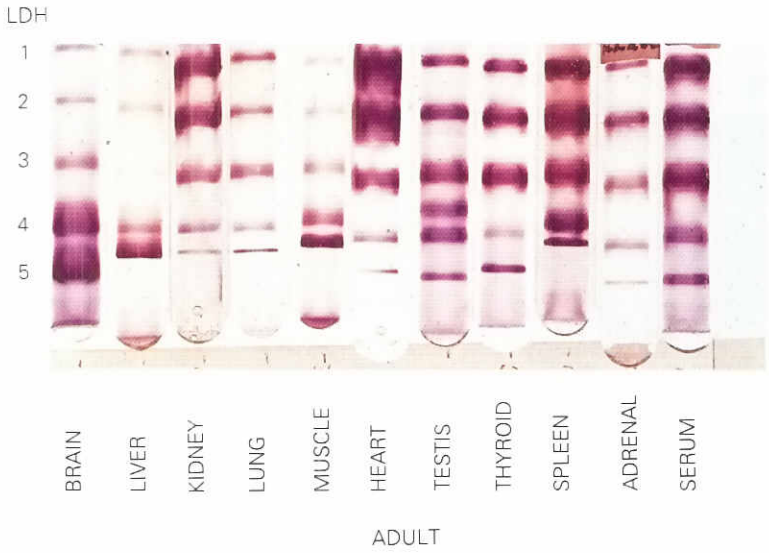

Fig. 5. Lactate dehydrogenase (LDH) isoenzyme patterns in the cytosols of adult human organs. *LDH isozymes 1 through 5 are indicated at the left margin. The lanes starting from the left contain cytosols from 1, brain; 2, liver; 3, kidney; 4, lung; 5, muscle; 6, heart; 7, testis; 8, thyroid; 9, spleen; 10, adrenal gland, and 11, serum.*

LDH-isoenzyme patterns of the fetal heart from this series of spontaneous abortions show retardation in the development of the isoenzyme LDH1 and LDH2, which are rich in H monomers.

Serum proteins change during teratocarcinogenesis in the mouse

Embryo-derived retransplantable murine teratocarcinoma (Solter *et al.*, 1970) are malignant tumors composed of malignant stem cells called embryonal carcinoma cells (Pierce, 1967), and cells undergoing differentiation and giving rise to various somatic tissues. One of these teratocarcinomas was restricted in its differentiation capacity solely to neuro-ectodermal derivatives (Damjanov *et al.*, 1971, 1973). The neural and glial cells formed in this tumor were mostly immature and incompletely differentiated cells, although mature neural and glial cells, as well as intermediate cells could also be found. In spite of the fact that the tissue organization is rather primitive, its protein extract in the 100,000 g supernatant contains the acidic, fast migrating anodic protein bands characteristic for the brain (Fig. 6). Although not all the protein bands of an adult brain extract could be demonstrated in the tumor growing on the hind leg, there is a remarkable similarity between the two tissues, when examined electrophoretically (Damjanov *et al.*, 1973).

How the host protein synthesizing machinery reacts to the neoplastic growth of embryonal carcinoma transplanted subfascially into the hind leg was the next problem. The soft, brain-like tumor described above, named TR-17, composed exclusively of embryonal carcinoma cells and neural elements, was allowed to develop for various time durations. The animals were sacrificed after 10, 19, 25, 30, 35, 40, 45, and 50 days, their blood collected and sera prepared together with the serum of the control: the healthy C3H mouse. Serum proteins were analyzed electrophoretically under nondenaturing conditions (Fig. 7). The comparison of serum protein patterns between the control mouse and the mice developing neurogenic teratocarcinoma displayed profound time-dependent changes. These changes in the serum compartment might represent the reflection of the immunological and molecular mechanisms of the host in the defense against embryonal carcinoma. The

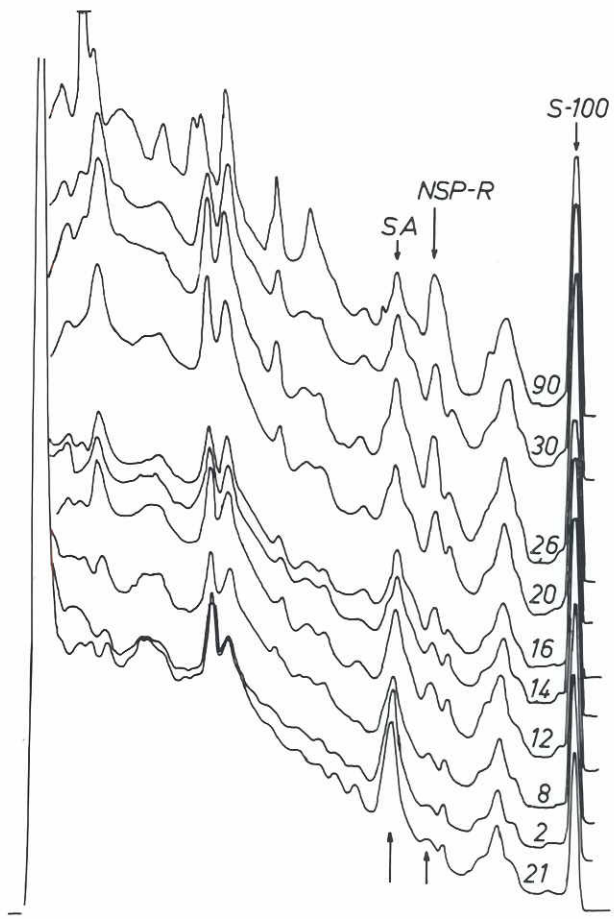

Fig. 6. Soluble proteins of the rat brain gradually change during postnatal development, starting from the 21-day-old fetus, up to the age of 90 days post partum. *The prominent brain-specific acidic protein peaks include: S-100, neuron-specific protein (NSP-R), and a number of others all travelling faster than the serum albumins (SA).*

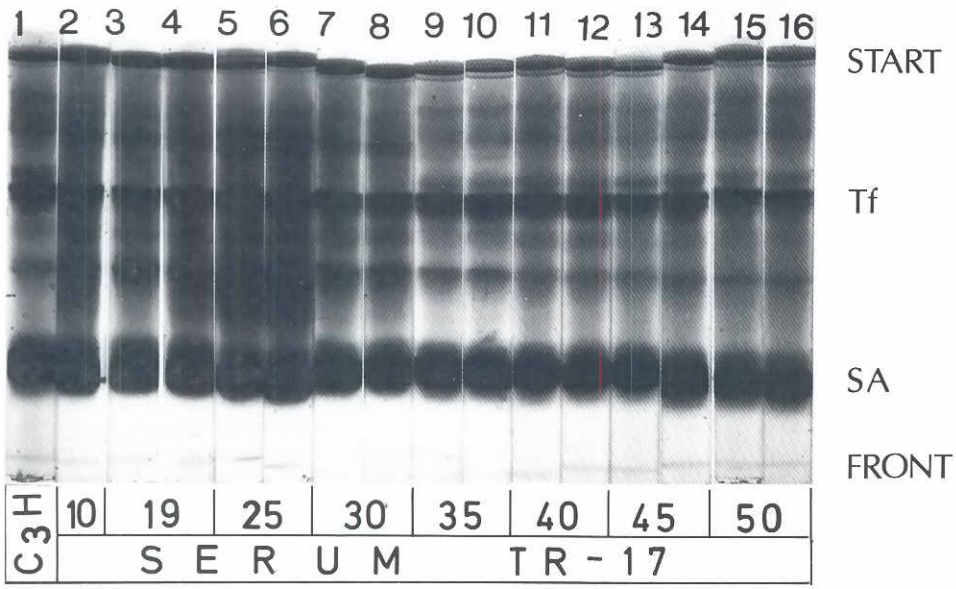

Fig. 7. Time course of serum protein changes in the mice bearing embryo-derived retransplantable neurogenic teratocarcinoma (TR-17). (1) *Healthy C3H mouse serum as control.* **(2 to 16)** *show the time-dependent serum protein changes after tumor had been grown in the host for 10, 19, 25, 30, 35, 40, 45, and 50 days.*

deviation from the normal serum protein pattern is already evident on day 10 of embryonal carcinoma growth. It develops further to day 19 and culminates on day 25 of teratocarcinogenesis. By day 30 (Fig. 7) the pattern very much regains the shape of the normal pattern, except for the protein band migrating in the immunoglobulin region, just behind the transferrins. The profound qualitative and quantitative changes of serum proteins are not restricted to changes in the immunoglobulin region. Even more drastic changes take place on day 25 in the whole region between serum albumins (SA) and transferrins (Tf), including total smearing among immunoglobulins.

Human leukocyte interferon inhibits crown gall tumor in plants

Interferon is a common name for a family of species-specific proteins which interfere with viral replication. In addition to its antiviral properties interferon may act as a cell modulator which causes a variety of biochemical changes in animal cells. They are the antiviral proteins which lack the virus specificity and are associated with DNA-dependent protein synthesis.

We wanted to find out whether human leukocyte interferon can protect plant cells from tumor transformation as it is capable of

Fig. 8. Crown gall tumors induced in potato tuber cells by *Agrobacterium tumefaciens*.

Fig. 9. Crown gall tumors can be induced on youngest fully developed leaves of *Kalanchoe daigremontiana* by wounding the cells and transforming them with T-DNA of *A. tumefaciens*.

Fig. 10. Human leukocyte interferon (hIFNalpha) suppresses the number of crown gall. (A) *Upper row: controls, potato tuber discs inoculated with* A. tumefaciens *displaying normal rate of tumor induction, 25 days after inoculation.* **(B)** *Middle row: experimental discs show suppressed number of tumors induced, when bacterial suspension was applied simultaneously with 2.14 x 10^5 units of hIFNalpha per ml.* **(C)** *Lower row: experimental suppression of tumor induction by simultaneous application of 1.07 x 10^3 units of hIFNalpha per ml.*

protecting plant cells from virus infection (Orchansky *et al.* 1982). We tested its influence on crown gall tumor induction on potato tuber cells (*Solanum tuberosum* L., Fig. 8) and the youngest fully developed leaves of *Kalanchoe daigremontiana* R. Hamet and K. Perr dela Bath. (Fig. 9).

Tumors were induced by *Agrobacterium tumefaciens*, and human leukocyte interferon (hIFN-alpha) was added to the bacterial suspension either at the moment of the inoculation or 48 hours later. The interferon content varied from 500 to 214,000 units per milliliter of the inoculum, and 50 microliters were applied per disc.

Fifty potato tuber discs were tested in each class and were incubated on agar plates. The number of tumors that appeared within 14 days was determined using the dissecting binocular microscope (Fig. 8). The number of tumors which developed on tuber discs inoculated with *A. tumefaciens* with simultaneous application of hIFN-alpha was significantly lower than the number of tumors on the discs inoculated with pure bacterial suspension (Fig. 10). This inhibitory effect was also observed when the IFN-alpha was applied 48 hours after the bacterial inoculation, *ie.* after the T-DNA has already been integrated into the plant genome (Table 1).

The tumors induced on damaged *Kalanchoe* leaves in the presence of interferon in the bacterial inoculum developed in different ways: (1) as rooty growths (differentiation of root), (2) as an unorganized tumor mass, and (3) as shoot-like tumors (differentiation of shoots) (Fig. 11, Table 2).

TABLE 1

THE EFFECT OF HUMAN LEUKOCYTE INTERFERON ALPHA ON CROWN GALL TUMOR INDUCTION IN POTATO TUBER TISSUE

Inoculum	Mean number of tumors per disc
A. tumefaciens B_6 804	27.25[a] + 2.213[b]
A. tumefaciens B_6 804 + hIFNalpha[c]	7.75 + 1.051
A. tumefaciens B_6 804 + hIFNalpha[d]	6.60 + 0.957

[a]mean of 10 replicates of 5 discs per dish
[b]standard error
[c]inoculum interferon content: 1.07 x 10^3 units per ml
[d]inoculum interferon content: 2.14 x 10^5 units per ml

TABLE 2

THE INHIBITORY EFFECT OF HIFNALPHA ON THE CROWN GALL TUMOR FORMATION ON KALANCHOE LEAVES

Inoculum	Number of tumors per 60 inocula	Tumor morphoogy Organ.	Unorgan.	Percent inhibition
B_6 804	60	6	54	0
B_6 804 + hIFN[a]	12	9	3	80
B_6 804 + hIFN[b]	4	2	0	93.3

[a]inoculum interferon content: 0.5 x 10^3 units per ml
[b]inoculum interferon content: 2.14x 10^5 units per ml

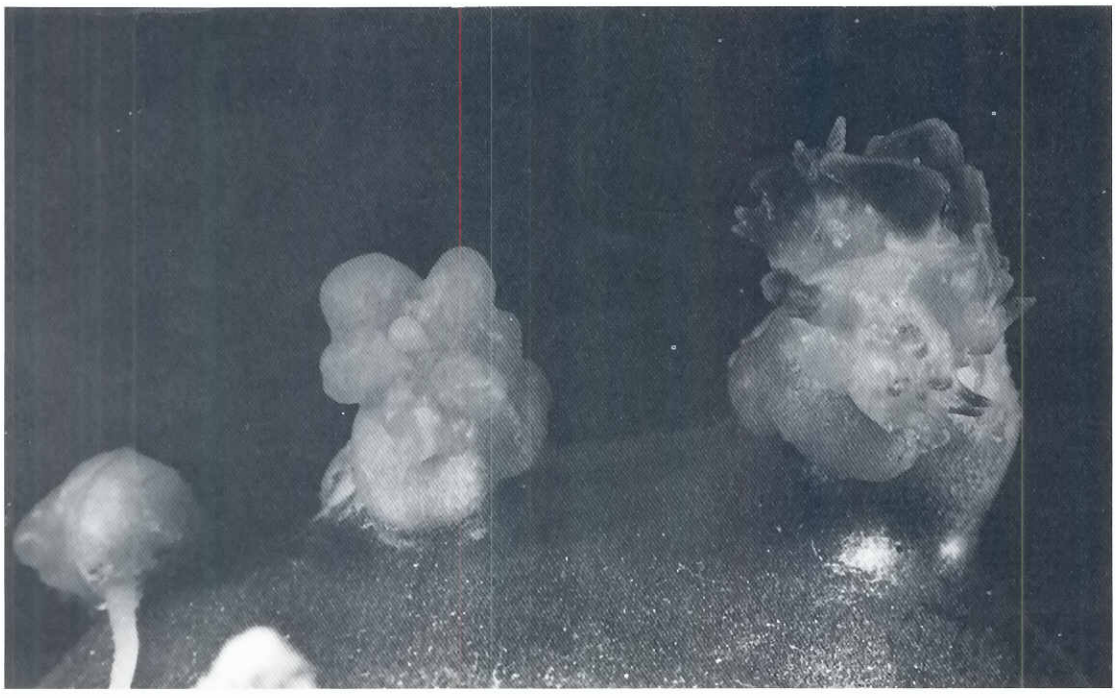

Fig. 11. Crown gall tumors developed on wounded leaves of *Kalanchoe daigremontiana* in the presence of hIFNalpha differentiated into morphologically different tumors: (A) *tumors producing roots;* **(B)** *undifferentiated white tumor masses, and* **(C)** *tumors producing shoots.*

Postimplantation rat embryo during *in vitro* cultivation modifies protein composition in spent media

The cultivation of early post-implantation rat embryo has advanced significantly in recent years. It was possible to study growth factor requirements of the developing embryo and it was clearly shown that whole rat embryos grew better in media supplemented with homologous serum than on the plasma clots (New, 1966). Some unknown factors from the rat serum added to the minimal essential medium were essential for normal development. Only a few studies attempted so far to analyze these factors, and some have suggested that protein fractions were depleted from the spent media, possibly utilized by the embryos cultured (Klein *et al.* 1978; Priscott *et al.* 1983).

TABLE 3

CONCENTRATIONS OF TOTAL SERUM PROTEINS AS MEASURED BY THE LOWRY METHOD

Origin of serum	Protein concentrations (g/L, mean + standard error)	N
Healthy individuals (controls)	74.6 + 8.4	97
Bearers of bennign neoplasia	79.5 + 6.7	178
Bearers of cancers	80.2 + 9.1	104

The modified organ culture of 9.5-day-old embryos was cultivated for 14 days in Eagle's MEM supplemented with 50% rat serum (Skreb and Crnek, 1980). The medium was changed every second day throughout the experiment, and the spent media were analyzed quantitatively and qualitatively for their protein content. Spent media, in comparison with controls, showed decreased amounts of proteins. Qualitative analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining of 117 samples of spent media indicated the depletion of some proteins from the spent media. According to the estimated molecular weights the polypeptides p132, p125, p100, as well as transferrins and albumins were depleted during the whole period of culturing.

Besides the observation of depleted proteins, starting from the fourth day onward the elevated levels of hemoglobin could be observed, as well as the polypeptide p74. This band is not visible in normal adult rat serum.

Protein parameters of gynecological malignancies in humans

Serum protein patterns were studied in 104 female cancer patients: 45 cervical cancers, 21 endometrial cancers and 33 ovarian cancers. One hundred and seventy-eight patients with benign neoplasia (59 cervical dysplasia, 72 endometrial fibromyoma and 47 ovarian cystadenoma), were also analyzed and compared with 97 healthy women as controls. Quantitative analysis by the Lowry method indicated higher total serum proteins in patients with cancer and benign neoplasia than in control sera (Table 3). The

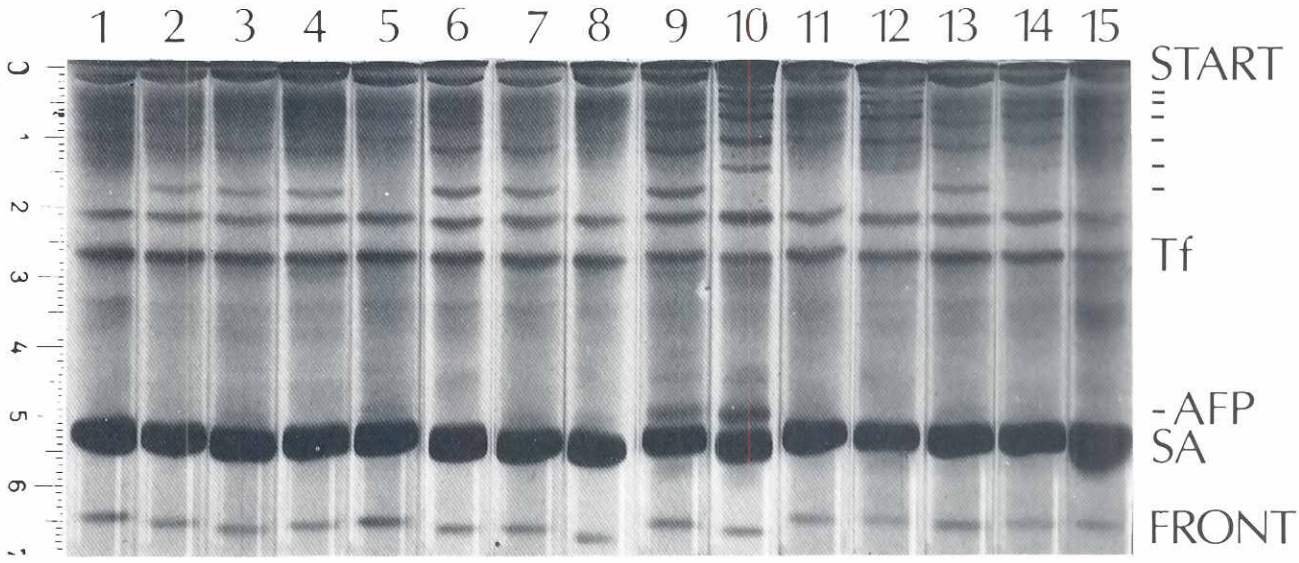

Fig. 12. Human serum protein patterns from patients with cervical cancer (2-6), *endometrial cancer* **(7 and 8),** *and ovarian cancer* **(9-15)** *in comparison with healthy control* **(1)**, *as revealed by PAGE.*

number of protein bands separated under non-denaturing (PAGE) and denaturing conditions (SDS-PAGE) indicated that they were roughly similar in healthy controls and in patients suffering from benign disease. The number of bands in sera from cancer-bearing patients is significantly higher (Fig. 12).

The cytosol proteins of cervical tissue (18 cancers, 24 dysplasias, and 18 normal) were analyzed in polyacrylamide gels, searching for the four most abundant protein components of the electrophoretic pattern. Under non-denaturing conditions of PAGE the protein having the electrophoretic mobility of 0.5 relative to serum albumins was the third most abundant peak in cervical cancer, while it was the fourth most abundant protein component in cervical dysplasia. In denaturing conditions of SDS-PAGE the polypeptide with a molecular weight of 35,000 (p35) was found only in cervical cancer. Therefore the data indicate that some neoplastic diseases in women may display a specific protein pattern both in the serum and in the cytosol compartment of the diseased tissue. It is interesting to note that Sugioka *et al.* (1985) have observed the appearance of the new polypeptide, p35 solely in well-differentiated hepatocellular carcinomas, during administration of 2-acetylaminofluorene to rats, and induction of hyperplastic nodules and hepatocellular carcinomas. Electrophoresis, particularly the two-dimensional systems, evidently offers the method for monitoring sequential protein changes during chemically-induced neoplasia, and with improvements for high reproducibility of separations, it will allow comparison among different samples, even for very subtle changes in the pattern (Wirth *et al.*, 1986, Yoshida *et al.*, 1991.)

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