T CELL-INDUCED NORMALIZATION OF TRANSFORMED MALIGNANT FIBROBLASTS: A LYMPHOKINE WITH NORMALIZATION FACTOR ACTIVITY

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Virtually all research into the effect of the immune system on malignant cells has been devoted to the study of cytostatic and cytotoxic effects. However, besides the function of elimination of foreign antigens, immunocompetent cells can also produce noncytotoxic regulatory effects. These include, in particular, the differential action of T cells on normal [5] and their normalizing effect on transformed malignant [9] hematopoietic cells. The writers showed previously [1] that spleen cells (SC) of C3H mice can induce normalization of syngeneic transformed malignant fibroblasts of the L-929 line (L-cells, LC) and that nonadherent cells are responsible for this phenomenon. In the presence of SC, stable normalization of the morphological, biophysical, and proliferative characteristics of LC was observed. The aim of this investigation was to discover lymphocyte populations responsible for the normalizing action of SC and to determine whether this effect is mediated through a soluble factor (factors).

EXPERIMENTAL METHOD

SC from C3H/Sn mice were separated into populations. Adherent cells (AC) were eliminated on plastic Petri dishes after incubation of SC in medium 199 containing 20% of embryonic calf serum for 40 min. The population of nonadherent cells was subjected to further successive fractionation by the panning method [10]: B lymphocytes (BL), or Ig^+-cells, were eliminated with the aid of the IgG-fraction of rabbit antiserum against mouse Ig (generously provided by the staff of the Lymphocyte Differentiation Laboratory, Institute of Immunology, Ministry of Health of the USSR); T lymphocytes (TL), or Thy 1.2^+-cells, were obtained with the aid of anti-Thy 1.2 monoclonal antibodies, the product of a G-4 hybridoma (generously provided by the staff of the Immunochemistry Laboratory, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR). Cells remaining in suspension after the panning procedure were classed as NUL cells (OC).

To obtain supernatants, SC in a concentration of 3×10^6 cells/ml were cultured in medium 199 with 10% bovine serum for 24, 48, and 72 h. Fractions of lymphokines were isolated from supernatants of 24-h serum-free cultures of SC. Methods of chromatography, used previously to isolate mesodermalizing differentiation factors [6], were used: chromatography on hydroxyapatite, gel filtration, electrophoresis.

The normalizing effect of lymphocytes, supernatants, and their fractions was tested by the use of a culture of LC as the test system. LC were introduced into flasks beneath penicillin at the rate of 0.7×10^5 cells and the same number of lymphocytes was added to them. The total volume of medium was 3 ml. When the effect of the supernatants was tested, they accounted for 50% of the volume of the culture medium. After culture for 72 h changes in the phenotype of LC were estimated, by comparison with intact control cultures, by studying the following characteristics: the transmembrane potential (TMP), by

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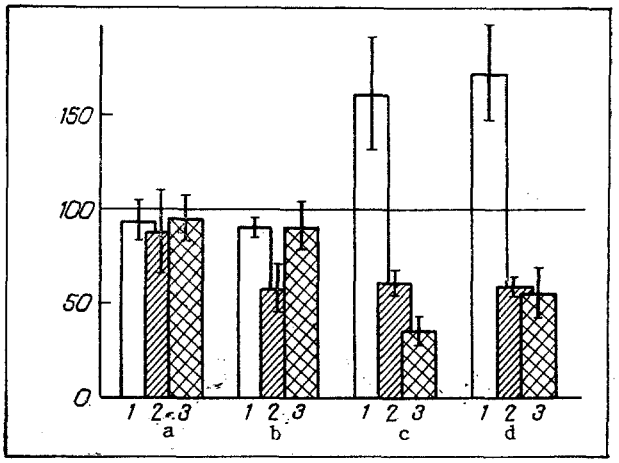

Fig. 1. Effect of separate SC populations of C3H/Sn mice on characteristics of LC culture. LC were cultured in the presence of AC (a), BL (b), TL (c), and OC (d). Ordinate, relative values (in %) of TMP (1), proliferative activity (2), and number of E-rosette-forming LC (3), compared with control (100%). Control consisted of a culture of intact LC.

the voltage-sensitive fluorescent probe method [2, 7], ability of the LC to form rosettes with sheep's red blood cells by the E rosette-formation method, usually used to detect TL [4], and proliferative activity as judged by incorporation of ^{3}H-thymidine.

The stability of the normalization effect was estimated by studying the same characteristics of LC, when cultured with lymphocytes or supernatants after the 1st-5th passage. In the case of cultivation with lymphocytes they were eliminated from the LC cultures by fractionation during passage by centrifugation in a Ficoll–Verografin density gradient (ρ = 1.056 g/cm^3). Supernatants of these modified LC (MLC) were obtained after culture of MLC for 72 h in flasks under penicillin (0.7 × 10^5 cells in 3 ml of medium).

EXPERIMENTAL RESULTS

To discover the population responsible for inducing normalization of LC, SC from C3H mice were fractionated by the panning method. After successive isolation of AC, BL, TL, and OL, the purity of the populations was analyzed. The number of Ig^+-cells in the BL-enriched population was 91 ± 1.3%, whereas the number of Thy 1.2^+-cells, in the TL-enriched population was 86 ± 1.8%. Thy 1.2^+-cells were present (19 ± 2.2%) among OC, but Ig^+-cells were absent. A study of the effect of each of the isolated populations on characteristics of the LC culture is illustrated in Fig. 1. Since malignantly transformed cells and, in particular, transformed fibroblasts are characterized by unrestrained multiplication, by increased expression of receptors for sheep's red blood cells [8], and also by a fall in the TMP level [3], it will be clear that normalization of the LC phenotype takes place only in the presence of TL and OL. AL had no effect on the parameters of LC studied, and BL changed only proliferative activity, and this alone, without the results of the other tests, cannot be regarded as a reliable criterion of normalization. Thus the effect of normalization of LC, induced by SC, is mediated by the presence either of TL and OL or of TL alone, for their percentage among OL is quite high.

The search for soluble factors with which normalizing activity may be linked was carried out by analyzing the action of supernatants of SC and fractions obtained by chromatography on them. Definite changes in the LC culture characteristic of normalization of transformed fibroblasts were observed in the presence of supernatants obtained after 24 h of culture of SC (Fig. 2). Supernatants of 48-h cultures gave a weaker effect, and supernatants of 72-h cultures changed only proliferative activity, and had no significant action on TMP or on the number of E-rosette-forming LC (ERFL).

To search for the lymphokine (lymphokines) with normalizing factor (NF) activity in supernatants of 24-h cultures of SC they were fractionated. The first stage of isolation of NF was chromatography on hydroxyapatite. It showed that all activity was eluted from the column in one peak, with sodium phosphate in concentration of 0.25-0.3 M. The active

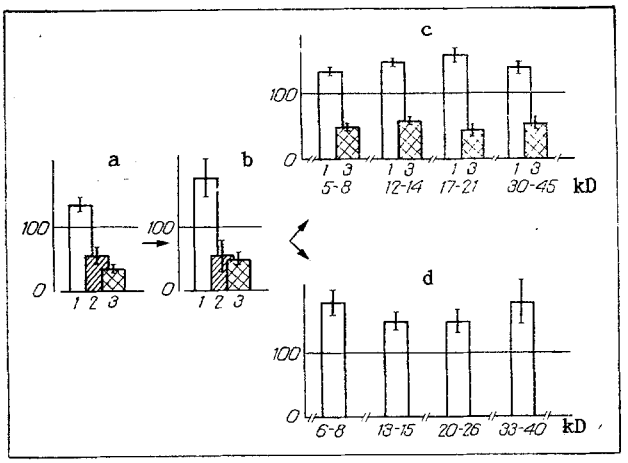

Fig. 2. Effect of supernatants of CS from C3H/Sn mice and lymphokines isolated from their fractions on characteristics of LC culture. LC were cultured in the presence of: a) supernatants of 24-h cultures of SC; b) active fraction isolated during chromatography of supernatant on hydroxyapatite, eluted from column with sodium phosphate in a concentration of 0.25-0.30 M; c) active fractions isolated during gel filtration; d) active fractions isolated during electrophoresis. Abscissa (c, d) molecular mass of fractions; ordinate, the same as Fig. 1.

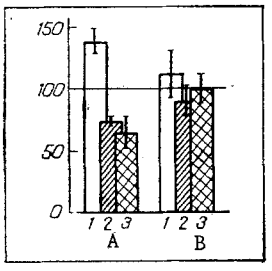

Fig. 3. Effect of supernatants of LC modified by lymphocytes (A) and intact LC (B) on characteristics of culture of transformed fibroblasts. Ordinate, the same as Figs. 1 and 2.

fraction caused the following changes in the characteristics of LC: TMP was raised by 70%, proliferation was reduced by 44%, and the number of ERFL reduced by 50% (Fig. 2). The active fraction thus obtained was further fractionated by gel filtration. In the course of testing of the fractions for their effect on TMP and on the number of ERFL, four peaks of activity were found with molecular masses (MM) of 5-8, 12-14, 17-21, and 30-45 kD. To determine the precise MM and discover the nature of the heterogeneity of NF, the fraction obtained after chromatography on hydroxyapatite was subjected to electrophoretic fractionation. Four active regions were discovered, corresponding to the same values of MM: 6-8, 13-15, 20-26, and 33-40 kD (Fig. 2). If electrophoresis was carried out after treatment of the original fraction with 2-mercaptoethanol, activity of NF was found only in the 6-8 kD fraction. It can thus be concluded that heterogeneity of NF for MM was connected with the ability of individual subunits to form oligomers. Thus as a result of the investigation it was shown that the normalizing action of lymphocytes on transformed fibroblasts may be mediated by a soluble factor capable of oligomerization.

Besides establishing the fact that lymphocytes have a normalizing action on transformed cells, it was necessary to characterize the phenomenon itself: its stability, the possible mechanisms of its realization. To assess the stability of the normalization effect, the study of the characteristics of the LC culture was repeated after removal of SC or their supernatants from it. Throughout several passages (from the 1st to the 5th) these modified LC (MLC) preserved the properties acquired during combined culture with lymphocytes, i.e., the normalized phenotype was inherited in several cell generations. To determine the causes

of stability of the phenomenon the effect of supernatants of MLC on characteristics of an intact LC culture was investigated. Supernatants of MLC obtained after culture for 72 h were found to have a significant normalizing action on LC (Fig. 3). Supernatants of intact LC had no such action. It can be concluded from the results that NF, synthesized by lymphocytes, can induce synthesis in LC of a new protein, analogous in its normalizing activity to lymphocytic NF, and which was probably responsible for stability of the normalization effect.

Besides normalizing activity relative to transformed malignant fibroblasts, the lymphokine which we isolated also possesses activity of differentiation factor (DF): it induces mesodermal differentiation of early embryonic cells in amphibians [6]. Combination of functions of NF and DF is characteristic also of other lymphokines [11], so that processes of normalization and differentiation can be regarded as related phenomena.

The main conclusions from this study can be summarized as follows. TL can induce stable (inherited) normalization of the phenotype of transformed malignant fibroblasts, and the normalization effect is realized by a lymphokine with MM of 6-8 kD, which can undergo oligomerization and possesses activity of both NF and DF. A wider interpretation of the data suggests that immunocompetent cells participate directly in the process of normalization of malignant cells, and one component of its regulation may be lymphokines. Lymphokines with NF activity may perhaps be physiological regulators of differentiation of nonlymphoid tissues. The stability of normalization induced by lymphokines demonstrates a new possible approach to the immunoregulation of tumor growth, based on the direct normalizing effect of TL and not on their cytotoxic effect.

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