EFFECT OF LYMPHOCYTES ON IN VITRO DIFFERENTIATION OF MOUSE L FIBROBLASTS

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The study of the properties and functions of immunocompetent cells in recent years has not only revealed the mechanisms of their interaction during the immune response, but has also established the role of the immune system in regulation of the function of nonlymphoid tissues. Features distinguishing cells of the immune system, such as their ability to circulate and to infiltrate the tissues, have already hinted at a role for them in numerous processes that may be indirectly connected with the immune response. For instance, lymphocytes have been shown to influence differentiation and proliferation of hematopoietic stem cells [6, 7], the functional role of interaction of lymphocytes with cells of the erythroid series has been discovered [4], and lymphocytes have been shown to participate in regulation of the regeneration and proliferation of connective tissue [1, 15]. The role of the immune system in processes of tumor growth is particularly interesting. This problem is being intensively studied and, because of this, the properties of immunocompetent cells infiltrating tumor tissues are being investigated. Immunocompetent cells have been shown to have a direct influence on the proliferative activity of tumors [12]. Meanwhile the part played by cells of the immune system in the process of differentiation of nonlymphoid and, in particular, of tumor cells, closely connected with proliferation, has received little study.

The aim of this investigation was to study the effect of immunocompetent cells on differentiation of transformed transplantable mouse L fibroblasts during their mixed culture.

EXPERIMENTAL METHOD

As the object chosen to act upon immunocompetent cells we used transformed mouse fibroblasts of the transplantable L line (clone 929), generously provided by the staff of the All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR. The L-cells were cultured for 72 h either in penicillin flasks or in plastic dishes 35 mm in diameter (Nunc, Nuclon Delta) with 3 ml of medium 199 containing 10% bovine serum. The number of L cells seeded was $5 \cdot 10^4$ and $1.5 \cdot 10^5$–$2 \cdot 10^5$, respectively. The cells were incubated in an Assab incubator with an atmosphere of 3% CO_2. Control samples contained only L cells, the experimental — a similar number of syngeneic or human lymphocytes. The syngeneic lymphocytes were spleen cells from C3H mice, from whose connective tissue the L line of cells was obtained.

Fractionation of the adherent cells (macrophages) and lymphocytes was carried out by preincubation of the spleen cells in plastic dishes for 30 min in medium RPMI-1640 with 10% embryonic calf serum (ECS). The action of supernatants obtained by culturing spleen cells in a concentration of $5 \cdot 10^5$/ml for 72 h in medium RPMI-1640 with 5% ECS, also was investigated. The volume of supernatant added to the L cells was 50% of the total culture medium. Human lymphocytes (mononuclear cells) were obtained from peripheral blood of healthy blood donors by centrifugation in a Ficoll–Urografin density gradient [9].

To estimate the degree of differentiation of the L cells morphological, proliferative, and biophysical criteria were used. The morphological characteristics comprised assessment of the degree of spread and dimensions of the L cells. To obtain these characteristics, before seeding of the L cells of some control and experimental samples coverslips were applied, and after the end of incubation these were removed and stained with hematoxylin and eosin. The preparations thus obtained were photographed and, by means of a projection apparatus, the

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outlines of the photographed fields of vision were transferred to paper of uniform density. No fewer than 100 drawings of cells from each preparation were made and cut out, grouped according to size, and weighed. The results were expressed in units of weight, correlating with the degree of spread of the cells on the substrate. Measurements were estimated by microscopic examination of a suspension of L cells with the use of a measuring grid. The degree of adhesion of the fibroblasts was determined from the time required to remove them from the substrate with trypsin. A number of standard proliferative characteristics, such as are usually adopted for estimating the degree of differentiation of cells of transplantable lines also were used: determination of density-dependent inhibition of cell growth or topoinhibition and contact inhibition.

In the study of these characteristics after culture, rinsing of the monolayer, and removal with trypsin, the L cells were separated from the remaining lymphocytes. For this purpose a suspension of L cells was centrifuged in a Ficoll—Urografin density gradient by the standard method [9]. L cells were left in interphase, and the mouse lymphocytes were lowered to the bottom of the tube. L cells of control cultures were subjected to the same procedure, but later both were seeded in equal numbers in penicillin flasks. In the study of topoinhibition, the medium was not changed, and starting from the 3rd day, the number of cells in the control and experimental samples was counted daily. To investigate contact inhibition, the medium was completely changed daily and the limiting seeding density was determined. As the biophysical criterion of the degree of cell differentiation, comparative measurement of the membrane potential (MP) was used. The following fluorescent probes were used for this purpose: 1-analinonaphthalene-8-sulfonate sodium — ANS (from Serva, West Germany) and 4-(p-dimethylaminostyryl)-1-methylpyridinium — DSM (Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR). The intensity of fluorescence of these probes changes in opposite directions with an increase in MP [5, 8], and for that reason, the use of the two probes in parallel gives a sharp increase in sensitivity of the method. The probes were added to L cells after the end of culture with lymphocytes. The incubation time of the L cells with ANS and DSM was 80 and 60 min, respectively, and the working dilution was 5 and 30 μM. The intensity of fluorescence was measured in an area of cell cytoplasm measuring 1.5 μ^2 by means of the Lyumam-I2 luminescence microscope with the FÉU-79 photoelectronic multiplier. The results were expressed as the ratio between the mean intensities of fluorescence of DSM and ANS: F. DSM/F.ANS. The results obtained by each method of assessment of the degree of differentiation of the L cells were subjected to statistical analysis. The significance of the difference between the mean values was determined by Student's test.

EXPERIMENTAL RESULTS

The use of morphological criteria to assess the degree of differentiation showed that when cultured in the presence of lymphocytes, the degree of spread and of branching of the L cells was intensified, thereby increasing their area of contact with the substrate considerably. During culture with both syngeneic and human lymphocytes the number of cells with a large area of contact with the glass increased (from 5 to 15%), and under these circumstances cells appeared whose area of contact with the substrate exceeded that of all the cells of the control cultures. Cells with a small area of contact with the glass (5 conventional units), accounting for more than 30% of the ordinary L cell population, were absent altogether. Further investigations showed that the increase in area of contact of the L cells with the glass during culture with lymphocytes was due both to an increased degree of spreading and branching and also to an increase in the true size of the cells and the degree of their adhesion: the mean diameter of the L cells, measured in suspension after removal from the support with trypsin, was increased by 1.5 times compared with the control, and the time required to remove them from the surface of the glass was 2.5 times longer than for the control cultures. Supernatants obtained by culturing lymphocytes, if L cells were added to the cultures, were unable to influence their morphological characteristics. Thus, on the basis of the generally accepted morphological criteria of differentiation of fibroblasts in vitro, namely spreading, branching, size, and also adhesive properties [2, 14], it can be concluded that under the influence of lymphocytes the degree of differentiation of the transformed fibroblasts increases. This phenomenon was not exhibited under the influence of soluble factors secreted by lymphocytes into the medium during incubation, but was due to contact or short-distance interaction between L cells and immunocompetent cells. Such a differential action of mouse and human lymphocytes points to the absence of species specificity of this phenomenon.

The proliferative potential of the cells is another criterion of their degree of differentiation. Normal cells migrate over the substrate and multiply until they form a continuous layer. This phenomenon is often described as density-dependent inhibition of cell growth or topoinhibition [11, 14]. A phenomenon similar to this is contact inhibition, which can be to some extent differentiated from topoinhibition by a frequent change of medium. The two phenomena are weakly expressed in transformed cells [11, 13, 14]. The investigations showed that preliminary culture with lymphocytes led to the appearance of topoinhibition when the number of L cells was half of that in the control cultures. The limiting feeding density, when assessing contact inhibition, was also significantly lower in the population of L cells cultured previously with lymphocytes. It can be concluded from the growth characteristics that after culture with immunocompetent cells transformed lymphocytes acquire the features of more differentiated cells. Preservation of these features after passage of the L cells is evidence that this phenomenon possesses a certain degree of stability.

For a more objective, precise, and rapid assessment of the degree of differentiation of L cells a biophysical criterion is used, namely measurement of MP with the aid of fluorescent probes, for we know that transformed cells have a low MP [3, 10]. After interaction of L cells with lymphocytes, a more than twofold increase was observed in the ratio between the mean intensities of fluorescence: F.DSM/F.ANS. The increased MP of the L cells was preserved after they were washed to remove lymphocytes and subcultured, confirming once again the definite level of stability of the differentiation phenomenon, induced by immunocompetent cells. Lymphocytes were responsible for the phenomenon, for changes in MP of the L cells were not found when they were cultured together with adherent cells (macrophages). The short-distance or contact character of the effect of the lymphocytes was confirmed once again by the absence of an effect of lymphocyte supernatants on MP of the L cells.

It can thus be concluded from the assessment of the morphological, proliferative, and biophysical characteristics of L cells that, on contact with lymphocytes, they exhibit features of more highly differentiated cells. This phenomenon is not species-specific, it is stable in character, and it is evidence of the real and potential capacity of the immune system to influence differentiation processes in proliferating nonlymphoid tissues.

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