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Interactions between Human Lymphoblastoid Cells and Human Fibroblast Feeder Layers in vitro

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Abstract. Lymphoblastoid (LB) cells interact in vitro with human fibroblasts. Cell complexes can be dissociated by trypsin treatment. Binding on fibroblasts is higher for Epstein-Barr virus-producer LB cells than for nonproducers, and depends on the origin of the fibroblastic cells. Stromal fibroblasts isolated from a metastatic lymph node tumor exhibited a highly increased potency of binding LB cells in comparison with fibroblasts isolated from normal breast epidermis or the primary breast tumor from the same patient. After a 10-hour interaction period, LB cells undergo abnormal divisions giving rise to the formation of 'mini cells'.

Introduction

Any change in the behavior of a cell interacting with other cells reflects an acquired property which may be correlated with morphological and functional alterations such as those occurring in malignant growth [1]. Efforts have been made to establish in vitro methods to study cell-to-cell interactions in relation to cancer [2–8].

Although the function of fibroblasts is not limited to that of a 'feeder layer' [9–16], their interaction with other cell types has not attracted extensive investigation. As fibroblasts are often used to support growth of lymphoblastoid (LB) cells [9, 10, 17], we examined and report here cell-cell interactions between these two cell types.

Materials and Methods

Cell Lines and Culture Methods

G-EP, G-BC and G-L finite cell lines originated from a 64-year-old female patient with breast ductal infiltrative adenocarcinoma, grade III and extensive lymph node metastases. Tissue specimens were aseptically removed during surgery. The patient had not received any chemotherapy or radiotherapy before surgery. G-EP cells were produced by trypsin dissociation from an explant of normal breast epidermis; they are fibroblast-like cells with normal karyotype and

exhibit intense contact inhibition of growth. These properties have remained stable until now, 22 months after their isolation. At the 19th passage, the doubling time of G-EP cells was 55–60 h. G-BC cells were produced by trypsin dissociation from a sample of the solid primary tumor. They formed multilayers consisting of a mixture of large fibroblast-like cells and medium size epithelial-like cells (about 10% of the cell population). A high proportion of aneuploid (about 60%) and polyploid (about 17%) cells was also observed. At the 11th passage, the doubling time of G-BC cells was 75–80 h. G-L cells were produced by trypsin dissociation from a sample of a metastatic nodule in a lymph node. The G-L cells were long spindle-like in appearance with irregular orientations on the glass or plastic surface. Periodically they formed large cell piles, even if they had not covered all the available surface. The proportion of aneuploid cells was about 20% and that of polyploid cells was about 14%. At the 20th passage, the doubling time of G-L cells was 55–60 h. G-BC and G-L cells stopped growing about 10 months after their isolation. Mouse embryonic cells were produced by trypsin dissociation of a whole 10-day embryo. For these present short-time experiments, all cell lines were cultured in either McCoy's or RPMI 1640 media (Flow Labs., Irvine, Scotland) supplemented with 15% fetal bovine serum (Flow Labs.).

Human LB lines EB-1 [18], EB-2 [19], GOR [20], Raji [21], P3HR-1 [22], HR1-K [23] and the marmoset LB line B95-8 [24] were grown in RPMI 1640 medium supplemented with 8% fetal bovine serum. All media contained 10^5 IU/l penicillin, 10^5 mg/l streptomycin and 2 mg/l amphotericin B. All cells were free of contaminations as shown by microscopic examination and by ^{3}H-thymidine labelling and autoradiography [25].

Procedure for the Study of Cell-to-Cell Interactions between Fibroblasts and LB Cells

Fibroblastic cells at a density of 1.5×10^5 to 3×10^5 cells/ml of cell suspension were allowed to attach and expand on glass coverslips placed in plastic tissue culture dishes for 24 h, in a humidified atmosphere of about 5% CO_2 in air, at 36.8 °C. The growth medium was then replaced by a homogeneous suspension of logarithmically proliferating LB cells at a density of 2×10^5 to 4×10^5 cells/ml of complete RPMI 1640 medium. This density was proved to be high enough to insure complete coverage of the fibroblast layer by a dense layer of LB cells. Before application, the suspension of LB cells was made homogeneous by means of gentle pipetting. Cells were allowed to interact for 2–24 h including the 20-min interval which LB cells required to settle down on the fibroblast cell layer. At the end of the incubation period, the coverslips were washed by shaking gently in three changes of physiological saline, fixed in methanol, air dried, and stained with buffered Giemsa pH 6.8. Stained coverslips were mounted on slides and examined and photographed with a Reichert light microscope.

Results

Interaction between LB and fibroblastic cells was determined by estimating the percentage of attached fibroblastic cells carrying on their surface LB cells. When one or more LB cells were bound to two or more fibroblasts, both or all fibroblasts were counted. LB cells are small and round, stain deep blue-purple with Giemsa and do not attach to glass or plastic surfaces, while fibroblasts stain light purple (nuclei) and light blue (cytoplasm) and attach to the substrate. When interaction time was limited to less than 10 min, only 0–2% of fibroblasts carrying on their surface LB cells were found, and this was independent of the types of fibroblasts or LB cells used. Since LB cells required no less than 20 min to settle down on the fibroblast layer surface, this value (0–2%) indicates that no cell-to-cell contact took place. Thus, the estimated values of cell-cell binding for interaction period of 2 h or longer actually show the potency of interconnections between LB and fibroblastic cells. Formation of cell-cell interconnections was rapid during the first hour of co-culture, but there was an insignificant increase in the number of cell hetero-complexes between 2 and 24 h.

Tables I and II show the results of two experiments on the binding of LB cells to fibroblasts, using fibroblasts in confluent and

Table I. Interactions between LB cells and confluent fibroblast layers

	Percentage of fibroblast-like cells carrying one or more LB cells on their surface[1]						
	Raji	GOR	EB-1	EB-2	P3HR-1	HR1-K	B95-8
G-EP T12, T19[2]	0–2[3]	0–2	0–2	0–2	0–2	0–2	95–100
G-BC T11	0–2	0–2	2–5	2–8	2–8	0–2	95–100
G-L T11, T20	15–20	6–10	24–31	65–72	67–78	63–71	95–100

[1] About 1,000 attached cells were counted in each case.
[2] T denotes passage number.
[3] Range among 25–30 optical fields in 2 replicate coverslips, under 500× magnification.

Table II. Interactions between LB cells and logarithmic phase fibroblast layers

	Percentage of fibroblast-like cells carrying one or more LB cells on their surface[1]						
	Raji	GOR	EB-1	EB-2	P3HR-1	HR1-K	B95-8
G-EP T12, T19[2]	0–2[3]	0–2	0–2	6–9	0–2	0–2	95–100
G-BC T11	2–5	2–7	5–10	7–10	2–5	5–12	95–100
G-L T11, T20	18–23	16–20	50–55	90–96	91–95	90–98	95–100

[1] About 1,000 attached cells were counted in each case.
[2] T denotes passage number.
[3] Range among 55–65 optical fields in 2 replicate coverslips, under 500× magnification.

logarithmic phases of growth respectively (fig. 1–6). Highly confluent cultures of G-EP cells do not incorporate tritiated thymidine into DNA and therefore they can be considered as stationary phase cultures; on the contrary, both G-BC and G-L confluent cultures incorporate tritiated thymidine, though at a significantly lower level than cultures in the logarithmic phase. These are referred to as 'confluent cultures'.

Both EBV producer and nonproducer LB cells show a very low affinity for binding to G-EP and G-BC cells, particularly when the latter are in confluency (fig. 1). In the logarithmic phase of growth, the affinity for interaction with G-BC cells is slightly increased for all LB cells, except P3HR-1. EB-2 cell affinity for G-EP cells in the logarithmic phase is also slightly increased. The degree of cell-cell binding was not altered when using fibroblastic cells in different passages. G-L cells exhibited an intense affinity for all types of LB cells used and this property was remarkably enhanced in the logarithmic phase (fig. 4, 5).

B95-8 cells exhibited the greatest affinity for attached cells, irrespective of cell type and phase of growth (fig. 3, 6). These marmoset LB cells formed large aggregates adhering to and completely covering the fibroblasts (fig. 6).

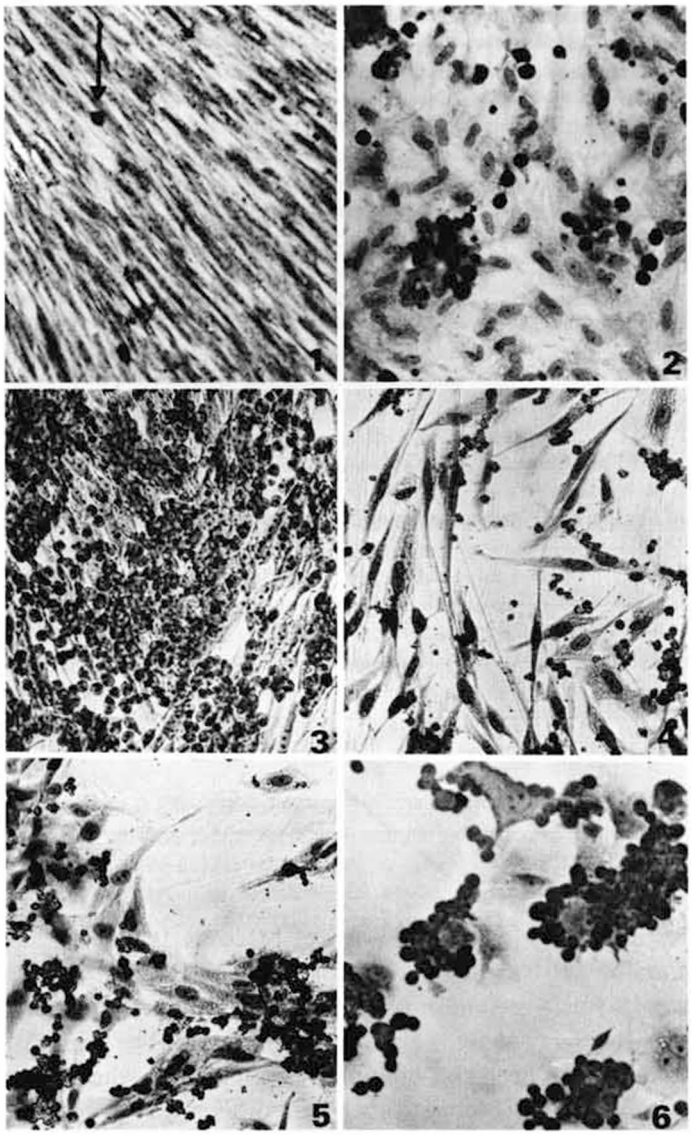

Fig. 1. Interaction of Raji cells with a confluent monolayer of G-EP fibroblasts for 2 h. The arrow shows a Raji cell. 100×. Giemsa.

Fig. 2. Interaction of EB-1 cells with a confluent monolayer of G-L cells for 2 h. 300×. Giemsa.

Fig. 3. Interaction of B95-8 cells with a confluent monolayer of G-BC cells. 200×. Giemsa.

Fig. 4. Interaction of EB-1 cells with a layer of G-L cells in logarithmic phase of growth. 150×. Giemsa.

Fig. 5. Interaction of P3HR-1 cells with a layer of G-L cells in logarithmic phase of growth. 150×. Giemsa.

Fig. 6. Interaction of B95-8 cells with a layer of ME cells in logarithmic phase of growth. 500×. Giemsa.

The number of LB cells bound to attached cells was dependent on the degree of agglutinability between the LB cells themselves. Homologous agglutinability for the LB cells used was increased in the order: Raji, GOR, EB-1, EB-2, P3HR-1, HR1-K, B95-8.

Following the 3-step washing in physiological saline, gentle shaking of the fibroblast X LB cell monolayers for 3 min in a solution of trypsin 0.1% in Mg^{++}, Ca^{++}-free Hank's BSS resulted in dissociation of the cell complexes and the dispersion of all the LB cells into the medium. The fibroblasts remained attached to the glass surface; fibroblasts re-

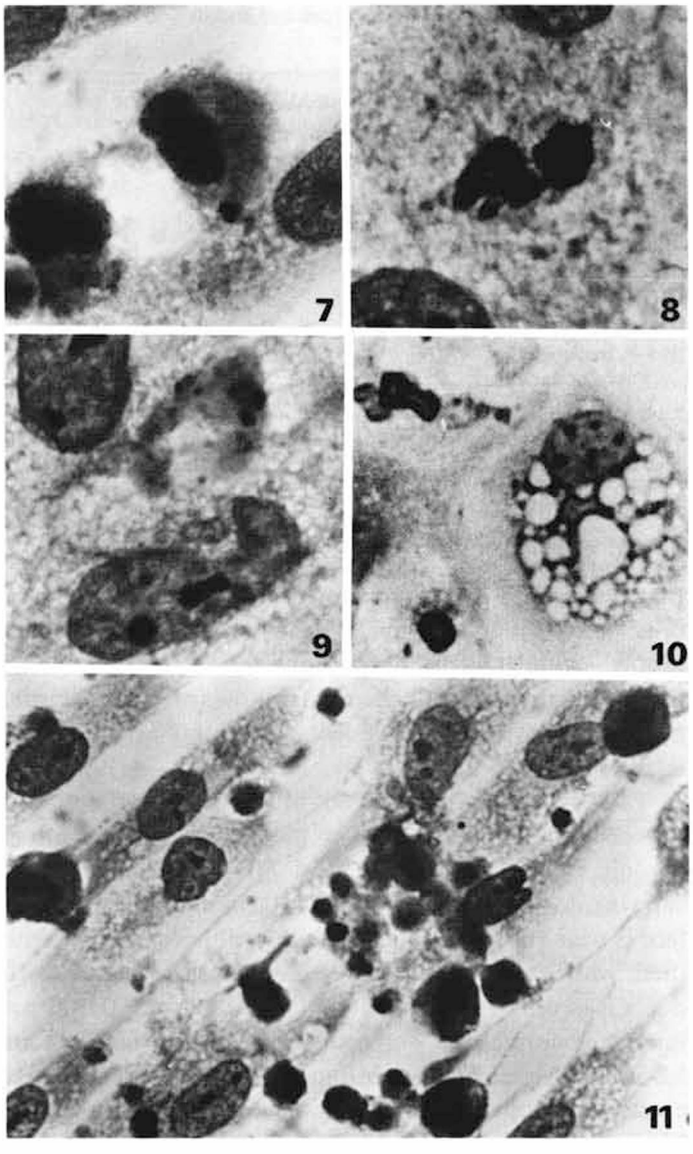

Fig. 7. Abnormal telophase of a dividing P3HR-1 cell showing formation of a micronucleus, after 10 h in co-culture with G-EP fibroblasts in logarithmic phase of growth. 1,500×. Giemsa.

Fig. 8. Nuclear fragmentation of a HR1-K cell after 10 h in co-culture with G-BC cells in logarithmic phase of growth. 1,500×. Giemsa.

Fig. 9. Cellular fragment of a Raji cell with several micronuclei (upper part) and abnormal nucleus of a G-EP cell (lower part), after 10 h in co-culture. 1,500×. Giemsa.

Fig. 10. ME cell showing extensive cytoplasmic vacuolation after 24 h in co-culture with EB-2 cells. 1,000×. Giemsa.

Fig. 11. Formation of 'mini cells' during in vitro interaction of Raji cells with G-L cells in logarithmic phase of growth, for 10 h. 1,000×. Giemsa.

quired a second 3-min treatment to detach from the glass surface. The liberation of LB cells was independent of the type of fibroblasts or LB cells used and the degree of cell-to-cell interconnections. The adhesion of LB cells to the fibroblast layer leads to cell-cell interaction between the two (fig. 7–11). After a 10-hour adhesion period, the following observations were made: (1) Nuclear abnormalities of the fibroblastic cells. The percentage of fibroblastic cells with nuclear fragmentation and abnormal nucleus shaping (fig. 9) ranged from 2.2 to 7.3%. (2) Appearance of 'mini cells' produced by abnormal division of

Table III. Interactions between human LB cells and nonconfluent monolayers of mouse embryonic (ME) cells

LB cells	Percentage of ME cells carrying one or more LB cells on their surface[1]	Percentage of ME cells with extensive cytoplasmic vacuolation
Raji	55–67[2]	0.5
GOR	100	2
EB-1	92–100	7
EB-2	90–100	5
P3HR-1	94–100	9
HR1-K	86–92	12
B95-8 (marmoset)	100	7

[1] About 1,000 attached cells were counted in each case.
[2] Range among 25–30 optical fields in 2 replicate coverslips, under 500× magnification.

LB cells (fig. 11). The origination of the 'mini cells' from LB cells is evidenced from the deeply staining nuclei and cytoplasm and from the high number of abnormal mitoses of LB cells (fig. 7–9). Experiments on 'mini cells' are continued.

Table III shows the results of interactions between human LB cells and mouse embryonic cells. The percentage of ME cells carrying one or more LB cells on their surface is near 100% for all EBV-producer lines used, while it is about 60% for the nonproducer Raji cells. After co-culture for 10 h, no nuclear abnormalities were noted in ME or LB cells. About 8% of ME cells showed extensive cytoplasmic vacuolation (fig. 10). This effect was 4–6 times lower in Raji than in the EBV-producer cells and was not observed in human fibroblastic cells.

Discussion

Cell contact is believed to be important for the regulation of growth, cell motility, cell differentiation and junction formation in malignancy [1, 26–28]. Since fibroblasts are found almost in every type of tumor tissue, it is justified to investigate interactions between fibroblastic cells and other cell types.

The main conclusions of this study are: (1) All types of LB cells show a higher binding affinity for G-L cells than for G-EP or G-BC cells in both confluent and logarithmic cultures. (2) Binding of LB cells to fibroblasts is enhanced when interaction takes place during the logarithmic phase of growth of fibroblasts. (3) The EBV-producer LB lines EB-2, P3HR-1 and HR1-K showed a remarkably high binding affinity for G-L cells. (4) LB X fibroblast cell binding occurs through a trypsin.

The results indicate the complexity of the interaction between fibroblastic and lymphoid cells and suggest that fibroblasts may develop functional surface properties for selective interaction with other cell types. The dissociation of the LB X fibroblast cell complexes by trypsin treatment indicates that cell-to-cell interaction occurs through a trypsin-sensitive protein. Differences in piling-up

and heterologous cell agglutinability properties between G-L and G-EP or G-BC cells may be attributed to quantitative or/and qualitative changes in surface proteins, which may be acquired due to the metastasis process. The functional role cell-to-cell interactions may play in metastasis has been stressed [1, 2, 29, 30]. Several authors investigating various cell systems have pointed out the significant role of the interactions of stromal fibroblasts with other cell types [3, 9, 10, 12, 13, 16, 28]. Differences in the agglutinability properties among EBV-producer cell lines indicate that different cell lines transformed with the same virus may develop different biological properties during in vitro cultivation. Human EBV-positive and EBV-negative LB lines have been shown to possess different and easily distinguishable surface glycoprotein patterns [31] which may affect agglutinability [32]. Thus, the differential intercellular affinities of each type of LB cells for G-EP, G-BC, G-L or ME cells could be attributed to the surface differences among LB cells. Evidence has been presented that human LB cells can aggressively invade and destroy normal human tissue [4] and *Deal* et al. [33] demonstrated the tumor-producing capacity of such a human LB cell line in pretreated mice.

In our experiments, we observed that normal human breast fibroblasts, to a significant extent, cause disintegration of the LB cells, giving rise to the production of enucleated 'mini cells'. This intrinsic type of 'cells' has been usually observed in tumor explants following a few days period in culture. From these and other unpublished results, we infere that 'mini cells' are produced by the disintegration of neoplastic cells. The biological properties and the role of these cells are unknown.

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