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Growth Promoting and Inhibitory Activities Of 3T3 and Other Cell Lines for Thiol-dependent Lymphoma Cells *in Vitro (40088).*

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A wide variety of growth factors exist for lymphoid cells in culture, and their study may contribute to an understanding of mechanisms responsible for growth control *in vivo* (1). Specific thiols and disulfides have an unusual position in this regard because although they profoundly modify the survival and promote growth of mouse cells *in vitro* they are either nonphysiological substances or are required in the medium in much greater than physiological concentrations (2).

The present experiments have been performed to study conditions *in vitro* which would make the provision of thiols unnecessary: they have used Lymphoma L1210(V), which is highly thiol dependent and L1210(A) a thiol independent variant (2, 3).

We now show that 3T3, L and other cell lines can support the growth of L1210(V) cells in the absence of thiol. The macrophage, studied by Nathan and Terry does not therefore in this respect have a unique relationship to the lymphoid cells (4). Cocultivation failed to produce a permanent conditioning of the medium, but nonetheless we demonstrate that growth promotion of the lymphoma cells is mediated humorally.

A further aspect of this system is shown by some cell lines which support lymphoma growth at low cell density but inhibit growth in confluent cultures.

Materials and methods. Reagents. 2-Mercaptoethanol (2-ME) and Concanavalin A (Con-A) were obtained from Sigma Chemical Company, St. Louis, MO.

Culture medium. Dulbecco's modification of Eagle's medium (DMEM) was used with air and 5% CO2 atmosphere (2). It was supplemented with 10% horse serum (Ho.S).

Animals. Normal $B_6D_2F_1$, male, 18–20 g, 7–12 weeks old were obtained from the Jackson Laboratories, Bar Harbor, ME.

Cells. L1210(V) and L1210(A) (2, 3) were maintained as ascites tumors in B6D2F1 mice. TP 5-9 a mineral oil induced malignant histiocyte tumor was kindly provided by Dr. Henry Azar. Mouse L cells were obtained from Dr. Karl Lanks. 3T3 Cells of various sublines were used. First were Balb/c cells obtained by Dr. Michael Howe from Microbiological Associates, Bethesda and carried in his laboratory for one year by splitting at intervals of 3 or more days. In the course of this time the cells lost contact inhibition and are considered "spontaneously transformed." Examination for mycoplasma sp. by Microbiological Associates, Diagnostic Labs. was negative. Other sublines, namely, Swiss 3T3 (ME) and Balb/c 3T3 were obtained from Dr. Ishwari Prasad.

Millipore diffusion chamber culture. Chambers were constructed and prepared as described previously and sterilized by ethylene oxide (5). All chambers were put up with 10^6 L1210(V) cells. They were placed on glass capillary pipettes (Microcaps, 100 microliters, Drummond Scientific Company, Broomall, PA), which were anchored with dental wax, 1 cm apart, on the bottoms of 60 × 15 mm Falcon tissue culture dishes (Becton, Dickinson and Co., Oxnard, CA) and immersed in culture medium.

Cocultivation of L1210(V) cells. (a) *With established tissue culture lines.* Supporting cells (3T3, etc.) were suspended after treatment with 0.25% trypsin, washed in saline, and inoculated into T flasks (Falcon, 25^2 cm) at densities of 1×10^5 cells and above. 3T3 Cells, 2×10^6, produced a confluent monolayer on attachment. The medium was changed 16–18 hr later, 1×10^6 lymphoma cells were added, and the cultures incubated for a further 48 hr. Lymphoma cells were then suspended by shaking and counted. They were easily distinguishable by their size, rounded shape and the presence of refractile cytoplasmic granules.

(b) *With embryonic cells.* Eight millimeter BALB/c mouse embryos were fragmented by cutting and agitation and trypsinized as

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above. Single cell suspensions were obtained by sedimentation. Monolayer cultures were then prepared and subcultured three times before use with lymphoma cells.

(c) *With macrophages.* Aliquots of $B_6D_2F_1$, nucleated spleen cells (2×10^7–2×10^8 in each experiment) were inoculated as described above in 4.5 ml medium. After 24 hours the flask surface was washed and the medium replaced. The density of adherent cells in all flasks of an experiment was then checked for uniformity by counts of the average number of cells in five high power fields; in no case was variation more than 10%. The absolute number of cells in one flask was determined by counting the suspension produced by thorough scraping with a rubber tipped rod. Adherent cell counts in different experiments varied from 5×10^5 to 5×10^6, and 98% of cells phagocytized carbon (four experiments). After these determinations were made, 5×10^6 L1210(V) cells were added and they were recounted 48 hours later.

Similar procedures were used with peritoneal macrophages.

Results. All standard tissue culture cell lines tested and also mouse embryo cells were able to support the growth of L1210(V) cells (Fig. 1).

3T3 Cells of different sublines were effective, irrespective of whether they showed contact inhibition or were transformed. Inocula of 5×10^5 3T3 cells per culture were optimal and 1-2 divisions of the lymphoma cells occurred within 48 hr.

When L1210(V) cells were stimulated to vigorous growth, addition of 2-ME increased the rate of lymphoma cell division only minimally. Thus, 2.00 ± 0.03 divisions occurred in 48 hr in the presence of 10^6 SV-3T3 (ME) cells. With added 2-ME, the corresponding figure was 2.25 ± 0.02.

In a single case, that of a BALB/c 3T3 subline which had lost contact inhibition during continuous passage ("spontaneous transformation") there was a notable dependence on population density. Two hundred thousand of these cells per culture failed to promote growth of lymphoma cells. At 4×10^5 cells, however, one division was produced but 2×10^6 cells were again ineffective. However, although no division of lymphoma cells occurred, with this inoculum more than 90% of the latter cells were viable at 48 hours. Strain L cells showed a similar density relationship; the optimal density was 1×10^5–2×10^6 cells per culture, and over this figure growth promotion diminished.

These results were compared to other findings with macrophage cultures (Fig. 2), which have previously been shown to support the growth of mouse lymphoid cells (4, 6). At inocula of 5×10^5 feeder cells, macrophages produced approximately the same degree of growth promotion of lymphoma cells (1.0–1.5 divisions) as did the other cell lines tested. But as the cell density of the macrophages increased, lymphoma cell growth became increasingly rapid, up to a level of 2.8 divisions at 2×10^6 cells. At this density, growth was at least equal to that produced by optimal concentrations of 2-ME (2.28 ± 0.14 divisions). Macrophages of various sources and a histiocyte cell line (TP 5-9) derived from a tumor which followed mineral oil carcinogenesis were all strong growth promoters, but thioglycollate stimulated peritoneal cells at 2×10^6 per culture seemed the most effective of

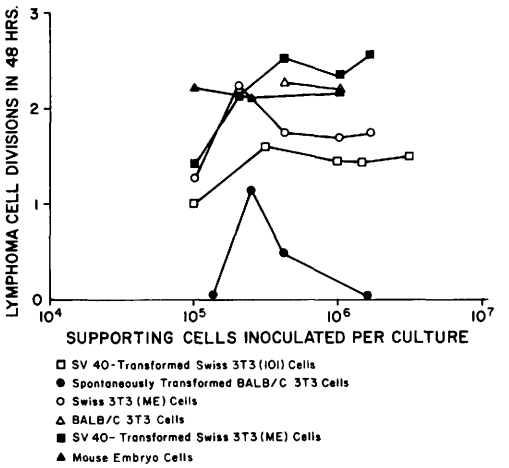

FIG. 1. Multiplication of L1210(V) lymphoma cells cocultivated with various cell lines in the absence of thiol compounds. Assays of growth promoting activity of feeder cells were performed in several experiments. In each of these controls were set up (a) negative-L1210(V) cultured alone in unmodified DMEM: no cell division occurred. (b) positive-L1210(V) cultured alone in DMEM + 5 μM 2-ME: 2.28 ± 0.14 cell divisions resulted. To ensure that the results obtained were not due to trypsin carried over by the feeder cells, this enzyme was added in concentrations of 0.001%–0.02% to culture media. L1210(V) cells inoculated into these in the absence of feeder cells failed to divide unless 2-ME was present.

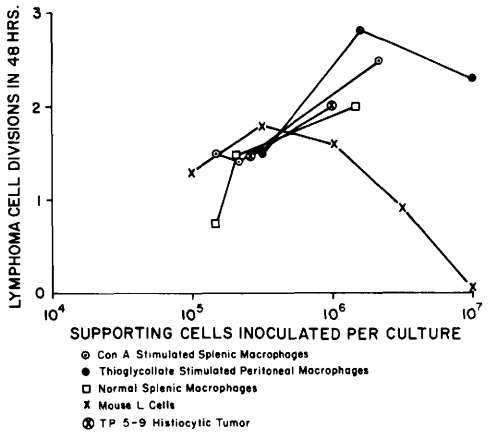

FIG. 2. Multiplication of L1210(V) lymphoma cells cocultivated with macrophages from various sources and L cells in the absence of thiol compounds. Methods as in Fig. 1.

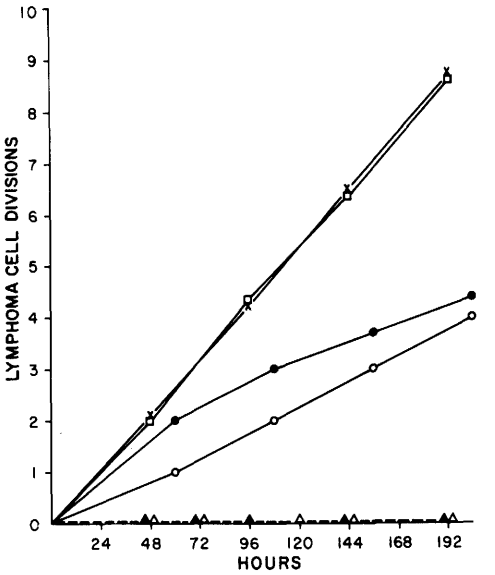

FIG. 3. Cumulative divisions of L1210(V) cells, under various conditions. ×, 5 μM 2-ME, no feeder cell. ▲, No thiol, no feeder cell. □, No thiol, thioglycollate-stimulated peritoneal macrophages (2×10^6 per culture). ●, No thiol, Con A-stimulated splenic macrophages (2×10^6 per culture). ○, No thiol, normal splenic macrophages (2×10^6 per culture). △, No thiol, L1210(V) cells grown for 48 hr with thioglycollate-stimulated peritoneal macrophages, then placed in new flask without feeder layer.

any cell type examined.

In these experiments, therefore, macrophages gave only growth promotion and not inhibition. They were therefore chosen for further experiments to examine growth promoting mechanisms, and "spontaneously transformed" BALB/c 3T3 at high density for inhibitory effects.

Mechanism of growth promotion. Supernatants were removed from 24, 48 and 72 cultures of macrophages (5×10^5 to 1×10^7 cells) which had been shown to provide optimal growth of L1210(V), and they were inoculated with lymphoma cells (1×10^6/ml). After 48 hr lymphoma cell recovery was 45% to 100% of the inoculum, in 12 experiments, but supernatants were in no case able to sustain growth. On the other hand, when 5 μM 2 ME was added to the supernatants, the lymphoma cells multiplied (1.6 ± 0.04 divisions in 48 hr).

The possibility that macrophages would only condition the medium in the presence of L1210(V) was then examined. It was observed that once mixed cultures of macrophages and lymphoma cells were established they persistently supported lymphoma cell growth (Fig. 3). The macrophages remained attached while the lymphoma cells in suspension proliferated. At 48 hour intervals, part of the medium with suspended lymphoma cells was removed and fresh medium was added to bring the lymphoma cell density to that of the original inoculum (1×10^6 cells/ml). On removing the medium with suspended cells 48 hr after changing and transferring to a fresh flask, no increase in cell number occurred, unless, as before, thiol was added (Fig. 3).

These experiments made it necessary to determine whether direct contact between lymphoma cells and macrophages or other cells was necessary for growth and this was examined by the use of diffusion chambers.

Diffusion chamber experiments. Petri dishes were plated with macrophage or other cell suspensions as in the preceding experiments, after 48 hr the medium was replaced and diffusion chambers containing known numbers of lymphoma cells were inserted. The chambers were positioned about 1.5 mm above the feeder cell layer.

When the diffusion chambers were removed after 72 hr, it was seen that macrophages had caused proliferation of the lymphoma cells (1.3–2.6 divisions); SV40 transformed 3T3 cells were also effective, but in the experiment performed less so than mac-

rophages (1.4 versus 2.6 divisions). With both cell types viability of the lymphoma cells was markedly increased. (Table I).

Again, supernatants removed from the macrophage culture before or after addition of the lymphoma cells failed to support growth unless thiol was added.

With other experimental work using diffusion chambers, objections have been raised that projections of cell processes through the pores of the membranes could be responsible for the effects observed (7). In the present experiments not only was fine porosity membrane used (0.1 μm), but the membrane was 150 μm thick and it was more than 1 mm from the macrophage layer.

These experiments indicate therefore that the feeder cells can affect lymphoma cell growth through humoral factors.

Cause of the failure of growth promotion by "spontaneously transformed" BALB/c 3T3 cells at high density. To test whether the failure of these cells at high density to support the growth of L1210(V) cells was related to or separate from their thiol replacing role, a subline, L1210(A) which is thiol independent *in vitro* was examined. Like L1210(V) this subline completely failed to grow in contact with 3T3 cells at high density nor did it do so in supernatant medium (Fig. 4). This observation and the inability of added thiol (to 50 μm 2-ME) to restore growth indicated that growth failure in this supernatant was distinct from thiol dependency.

Dilution of supernatant with an equal volume of fresh serum-containing medium permitted some growth (1.6 cell divisions in 48 hr compared with 2.5 cell divisions in com-

TABLE I. GROWTH PROMOTION BY SPLENIC MACROPHAGES SEPARATED FROM LYMPHOMA CELLS BY THE MEMBRANE OF A DIFFUSION CHAMBER.

	Cell type on Petri dish	2-ME (μM)	L1210(V) cells inoculated into diffusion chamber (×10⁶)	L1210(V) cells recovered at 72 hr (×10⁶)			Cell divisions
				Viable[a]	Dead	Total	
Expt. 1	None	0	1.0	0.2	0.3	0.5	0.0
	None	5	1.0	5.6	0.5	6.1	2.5
	1.3×10^6 splenic macrophages	0	1.0	2.4	0.1	2.5	1.3
Expt. 2	None	0	1.0	0.2	1.4	1.6	0.6
	None	5	1.0	6.2	0.6	6.8	2.7
	4×10^6 peritoneal macrophages	0	1.0	6.0	0.3	6.3	2.6
	5×10^6 SV40-transformed 3T3 (ME) cells	0	1.0	2.7	0.2	2.9	1.5

[a] Viability determined by trypan blue exclusion. Peritoneal macrophages were obtained from BDF mice injected with 2 ml thioglycollate medium 4 days previously.

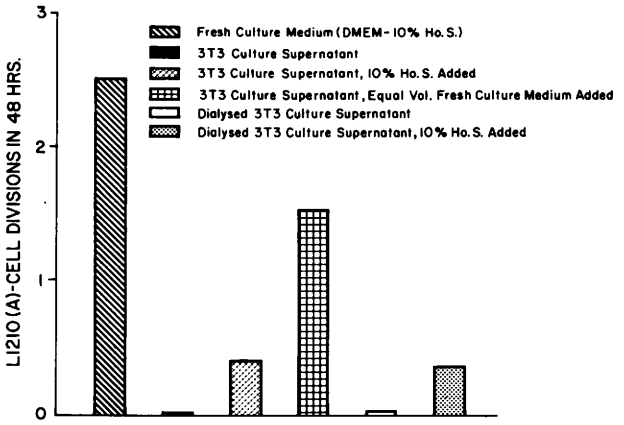

FIG. 4. Inhibition of growth of L1210(A) cells by supernatants from "spontaneously transformed" BALB/c 3T3 cells. 3T3 cells were inoculated at (4×10^7 cells) in 60 ml and incubated for 48 hr. Supernatant was removed at this time and aliquots were treated as shown, 1×10^6 L1210(A) cells/ml were then added and recounted after 48 hr. Dialysis was carried out against 100 volumes of fresh DMEM without serum.

pletely fresh medium). On the other hand, dialysis of supernatant against fresh Dulbecco's medium had no effect in restoring the ability of the medium to support growth. Fresh serum added to such medium had only a slight growth promoting effect (0.4 cell divisions in 48 hr). The latter two experiments indicate that in the present context, depletion of nutrients in the medium by 3T3 cells is of relatively minor importance in preventing lymphoma growth. It appears likely therefore that a nondialyzable inhibitor of growth of the lymphoma cells is produced by 3T3. This effect awaits further characterization.

Discussion. The experiments described show that a variety of cells, in addition to the macrophage, can produce conditions in culture which make the provision of growth promoting thiols and disulfides unnecessary for L1210(V) cells (Table I, Fig. 1–3). That humoral factors are involved is apparent from the diffusion chamber experiments (Table I), but surprisingly macrophages and other feeder cells are unable to condition the medium permanently.

A possible explanation is that growth promoting factor(s) from the feeder cells are unstable and must be continuously produced or reactivated. There are however, several other possibilities; the concentration of growth promoting factor in the external medium might limit its production by a tight feed back between utilization and production: alternatively feeder cells may be necessary for the inactivation of soluble autoinhibitory products of the lymphoma cells. Further experiments are required to permit a decision between these different mechanisms.

The experiments now described have shown another aspect of growth control mechanisms for lymphoma cells by the inhibitory action of higher cell densities of certain 3T3 and L cells, which appears to be mediated at least in the former case by a nondialyzable product(s). The putative inhibitor therefore may have either a high molecular weight or it may bind to protein in the medium. Similar inhibition has been reported by a factor(s) in supernatants of macrophage cultures of considerably higher density than those now used (8).

The possibility that the growth inhibition now observed was due to arginine deprivation as a result of contamination of 3T3 cells with mycoplasma was excluded by direct measurement using a Jeolco JLC-5AH analyzer; less than 30% of the initial arginine content of the medium was metabolized under the conditions of the experiments (9).

The significance of factors such as those now described for L1210 cells *in vitro* to growth control systems *in vivo* is uncertain. Clearly however, it is only by the use of simple *in vitro* systems that growth factors can be characterized and isolated. They will then be available for studies in systems of increasing complexity and finally in the whole animal.

Summary. The growth of thiol dependent cells of Lymphoma L1210(V) is supported by 3T3 and L cells as well as by macrophages *in vitro*. The supporting cells do not permanently condition the medium, but diffusion chamber studies show that growth promotion is mediated humorally. At high cell density inhibition of lymphoma cell growth can be observed in the supernatant from one of the 3T3 sublines examined, which appears to be due to a nondialyzable inhibitor whose effect is unrelated to thiol dependency.

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