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Interaction of Human Melanoma Cell Lines with Autochthonous Lymphoid Cells[1]

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INTRODUCTION

The cytotoxic effect of fresh lymphocytes on tumor cells *in vitro* has been reported by numerous investigators, one of the earliest being Humble *et al.* (1). The cytotoxic activity of fresh lymphocytes for primary cultures of tumor cells has been evaluated by counting the surviving tumor cells (2) or by calculating the percentage reduction in tumor cell colonies as compared to control primary cultures of tumor cells without added lymphocytes (3). Quantitative assays for studying interactions between fresh lymphocytes and target cells have been reported by Wilson (4–6). Studies of the cytotoxic action of fresh mouse lymphocytes on short term cultures of allogeneic tumor cells include those of Brunner *et al.* (7–8), Billingham (9), Jamieson *et al.* (10), and Alexander and Hamilton-Fairley (11).

Assays based on radioactive prelabeling of target cells with chromium-51 (12–14) or with ^{3}H-thymidine (15) are well established. Sinkovics and others have reported that cultured lymphoid cells are nonspecifically cytotoxic to allogeneic target cells and that this reaction cannot be "blocked" (personal communication, 16–19). We describe here an *in vitro* assay for tumor cell growth or death using long-term malignant melanoma cell lines as targets in culture with established autochthonous lymphoid cells. The assay is based on measuring extracted stain from

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surviving attached tumor cells in multiple cultures fixed at time intervals. This assay was correlated with time-lapse microscopy and electron microscopy to evaluate the cytotoxic effect of autologous lymphoid cells on malignant melanoma cells. The cytotoxic effect appears to be specific, and it can be "blocked" by certain autologous sera.

The culture of melanoma cells derived from mouse, hamster, and human tumor biopsy material has been reported by a number of authors (20–26), as have the techniques for establishing human lymphoid cell lines (27–28). This report describes preliminary experiments designed to evaluate cytotoxic interactions between autologous fresh lymphocytes and cultured lymphocytes with melanoma cells in mixed cultures.

METHODS

Initial Culture of Melanoma Cell Lines

Monolayer cell lines were established from malignant effusions and biopsies of solid tumors. The cells were freed from the stroma, either by mincing the tumor or by exposure to 0.25% trypsin. Cultures were initiated in 4-oz. glass bottles with either nutrient medium F-12 (29) or RPMI 1640 (30) medium supplemented with 10 or 20% heat-inactivated fetal calf serum (FCS) containing penicillin and streptomycin, and incubated at pH 7.0–7.3 as static cultures at 37°C. The initial cell population consisted of over 1×10^6 cells per ml. When the cells had grown nearly to confluency (several weeks to months), they were subcultured by scraping off about 50% of the monolayer with a rubber policeman. It was possible to subculture the cells weekly.

The peripheral blood was the source of hematopoietic cells, thus avoiding fibroblast contamination. One out of two lymphoid cell lines derived from patients with malignant melanoma become established and one out of five derived from normal donors, become established.

The morphology of the lymphoid and tumor cell lines was examined by light microscopy and electron microscopy. Karyotypic studies were performed to determine the chromosome constitutions (31). The HL-A antigen patterns were determined by fluorochromasia cytotoxicity method with NIH typing sera (32). Monolayers were tested for melanin production by means of Fontana staining. Melanin granules were observed in preparations of tumor cells grown on Leighton tube coverslips stained with a modification of the Fontana-Masson silver method (33). The 10% silver nitrate solution is diluted with an equal volume of distilled water and cleared by titration with 28% ammonium hydroxide. The preparations are stained for 2 hr at room temperature. Antibodies specific to cultured melanoma cells were detected in several autologous sera using the immunofluorescence method of Morton *et al.* (34).

Suspension lymphoid cultures were tested for immunoglobulin production (35). Many melanoma cell lines were tested for heterologous growth in month-old mice immunosuppressed by prior, total body irradiation (600 R) (36) for confirmation of their malignancy.

Cultures of melanoma cell lines or the lymphoid cell lines may be maintained in RPMI 1701 medium (but with HEPES reduced to 3 m*M*) (37), or GEM 1717

(38) supplemented with 10% FCS. Portions of the original cultures are frozen and stored in a cell bank (39).

Microdroplet Cytotoxicity Assay

The cultured autochthonous tumor cell-lymphocyte interactions were observed in Microtest II plates that consist of eight rows of twelve 0.4-ml wells (Falcon Plastics). The number of tumor cells was adjusted to 10^4 viable cells in 200-μl volumes for addition to each well. The cells were placed in the culture wells with Centaur Micropipettes (Centaur Chem. Co.). The plate was then sealed, and the cells incubated for 6–18 hr at 37°C in 7% CO_2 in saturated air to insure attachment of the tumor cells to the flat bottoms of the wells. The desired numbers of lymphoid cells in 200 μl of fresh medium were added to each well after aspirating the initial 200 μl of culture medium. The cultured lymphocytes were added to the wells in the ratio of 100, 50, or 20 lymphoid cells per tumor cell. The test plate was resealed and incubated at 37°C in the saturated CO_2 atmosphere.

Assays of tumor cell growth, inhibition of growth, and tumor cell death require that replicate wells be fixed at intervals of 10–12 hr by aspiration of the media and replacement with 100% methanol for 4 min at room temperature. After methanol fixation of the entire plate, the cells remaining attached in each well were stained for 4 min at room temperature with 400 microliters of a 5% Giemsa solution. Each plate was dipped quickly in five changes of cold distilled water to remove excess stain and then dried at 55°C for 30 min.

Color or black and white photographic prints were made of each plate for a permanent record. The optical density of each stained well was recorded and related to the number of stained cells by means of a densitometer attached to a microscope with a 10× objective and a 480-nm condenser filter.

A stain extraction method has also been developed for quantitation of the lymphocyte-target cell reaction in the microwells. The stain can be extracted from the cells in each well after agitation of the plate for 10 min at 37°C with 200 μl per well of 0.1 *M* glycine–HCl buffer at pH 1.8. The resultant samples were assayed in a colorimeter (660 nm; the absorption maximum of the extract) using a Technicon autoanalyzer system. These values were then related to the number of cells by comparison to standard extracts from known cell numbers.

The indirect quantitation of tumor cell death by the stain extraction method provided an objective assay. Visual cell counts of a large number of wells is time consuming and subjective. The densitometer method was inaccurate for measuring dense multicellular clumps.

Growth assays of the lymphoid cells were done with duplicate plates by removing 100 μl from each well, staining the cell suspension with 100 μl of 0.4% trypan blue, and counting the viable lymphoid cells with the aid of a cell counting chamber.

Time-Lapse Microscopy

Kinetics of the lymphocyte-tumor cell interactions were monitored with time-lapse phase cinemicrography. Cells or mixtures in RPMI 1701 medium with 10% FCS were inoculated into sterilized Rose chambers with a 0.5 ml capacity. Cell movement was recorded with an inverted Nikon phase contrast microscope equipped with a 37°C CO_2 incubator and a 40× objective.

Electron Microscopy

Samples for electron microscopy were pelleted by centrifugation at 800 *g* for 10 min and were immediately fixed and prepared for thin sectioning as described by Anderson (40). Sections were examined in a Siemens Elmiskop 1A electron microscope using a 20 μm objective aperture and operating at 80 kV.

RESULTS

Thirty melanoma cell lines have been established from the biopsies and effusions of 18 patients and, in one instance, from blood (41). In addition, 116 lymphoid cell lines have been established from 50 malignant melanoma patients. Six of the melanoma cell lines have matching autologous lymphoid cultures. In one instance, a fibroblast culture was also established from a tumor biopsy.

The malignant melanoma cell lines are comprised of comparatively large cells that contain melanosomes (Fig. 1). In general, melanin production appears to be

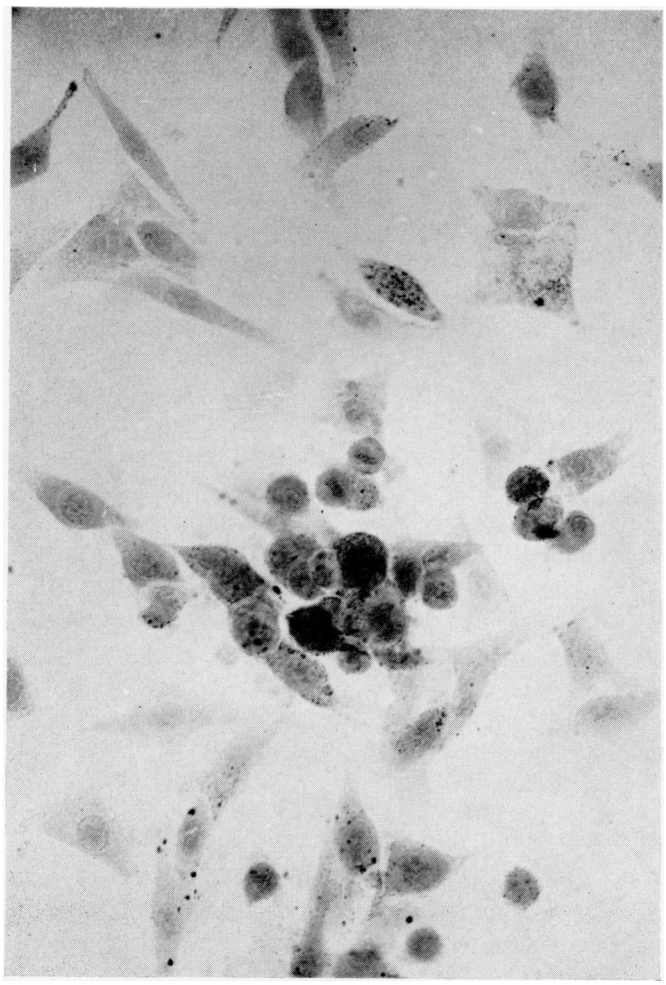


FIG. 1. Photomicrograph of RPMI M7821 melanoma cells grown on Leighton tube coverslips that have been stained by the Fontana reaction. Most of the cells seem to contain melanin.

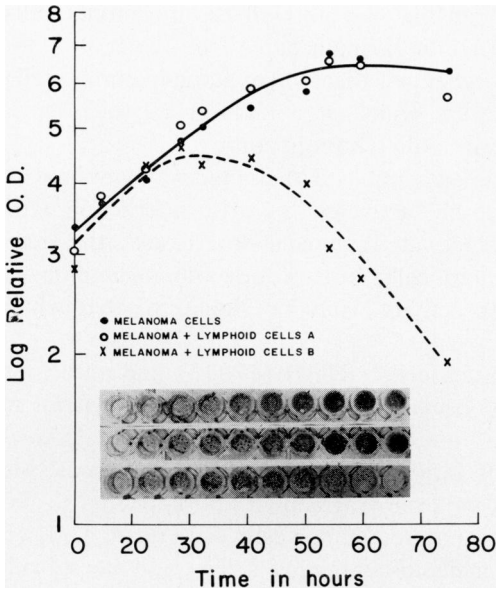


FIG. 2. The details of the semimicro assay are given in the Methods. The top curve shows tumor cell growth and similar growth (RPMI M7041) with one culture of lymphoid cells (RPMI 7491) (20/1) which evidently could not kill them. The dotted bottom curve shows the death of tumor cells which occurred when another culture of lymphoid cells (RPMI 7481) was added at the same ratio. The inserted photographs show the actual wells of the plate which was assayed here by means of a microcolorimeter. The stain can be extracted and quantitatively measured automatically (See Methods).

inversely related to the growth rate of the melanoma cells. Cultures in the stationary growth phase or those that are dying contain the greatest number of highly pigmented cells. Several cultures have maintained their ability to produce visible amounts of pigment for a period of six years. Tumors formed in irradiated mice also continue to produce melanin. All of our melanoma cell lines have abnormal chromosome constitutions ranging from a pseudodiploid state to the more common condition of polyploidy. Marker chromosomes have been observed frequently.

The lymphoid cell lines are all comprised of primitive or lymphoblast-like cells. All of them continually produce immunoglobulins. Most lymphoid cell lines contain predominantly normal diploid cells, and the cell lines appear to be stable for many months.

Antibodies to tumor antigens were detected in several autologous sera which reacted specifically with the membranes of cultured cells. Preliminary studies indicated that both the cultured human lymphoid and melanoma cells possess more detectable HL-A antigens than autochthonous fresh lymphocytes; perhaps these antigens are not exposed on the fresh cells. The results of a semimicro plate assay for cellular immunity are given in Fig. 2. Equal numbers of tumor cells (1×10^4) were dispensed into each well of a Falcon plastic Microtest II plate. The photographic insert shows the wells of such a plate. Each vertical row of cell cultures has been incubated and then terminated at intervals by fixing with methanol and staining with Giemsa. The top row contained melanoma cells (RPMI M7041) with medium only. In the second row an autochthonous lymphoid cell (cell line RPMI 7491) has been added to each well at a ratio of 20 lymphocytes to each melanoma

cell. This lymphoid cell line did not kill the melanoma cells, as the growth rate of the melanoma cells is indistinguishable from control cultures (top curve). The bottom row, however, shows that when autochthonous cells from a second cell line (RPMI 7481) were added at a ratio of 20 to 1, the tumor cells grew for about 30 hr, then began to die (bottom curve).

Preliminary experiments indicate that inactive lymphoid cells, similar to those depicted in Fig. 2, can be "activated" by prior interaction with live melanoma cells. Certain sera (heat-inactivated) appear to "block" the active cultured or fresh autochthonous lymphoid cells from killing the melanoma cells. This "blocking" of "lymphocyte killer activity" may be similar to that which has been reported by others (42–53).

Mixtures of melanoma cells (RPMI M7821) and autochthonous lymphoid cells (RPMI 7711) were observed by time-lapse, phase-contrast microcinematography. Figure 3 shows eight time intervals of the same field from the frames of such a film; after only 40 hr target cell destruction was obvious. Most of the melanoma cells had rounded up or even fragmented and appeared to be dead. The surviving cells were lymphoid in nature. Tumor cells were dead, as determined by the uptake of trypan blue. No melanin-containing cells could be subcultured from the cells in the supernatant media. Mixtures of autochthonous fibroblasts with the same lymphoid cells grew together continuously without evidence of growth inhibition or increased cell destruction. In one experiment the activated lymphoid cells also killed fresh autochthonous tumor cells.

Mixtures of melanoma cells (RPMI M7821) with autochthonous lymphoid cells (RPMI 7711) were observed at several time intervals by electron microscopy. After 60 hr the majority of melanin-containing cells were disintegrating and had lymphoid cells attached to them (Fig. 4a, b).

DISCUSSION

Only 1–10% of malignant melanoma tumor biopsies were established as permanent cell lines. The best source for successful culture were tumor cells that had become established in either pleural or peritoneal effusions. These tumor cells had apparently adapted themselves to a culture-like environment and were thus easier to culture *in vitro*. Biopsies obtained from such metastatic lesions generally had a more favorable growth rate than those obtained from primary tumors. The tumor cells remained in the primary culture flask for weeks up to 9 months before subculturing was possible. Then the cells grew with a doubling time of about 40 hr. Occasionally, fibroblast-like cells resulted from the original cultures. Scraping cells from the walls of the culture bottle seems to favor the growth of melanoma cells and inhibit the growth of fibroblasts. Attempts at adapting tumor cell lines to suspension culture environments indicated a selection for clonal growth. Such suspension cultures are not useful in the assays described.

Lymphoid cell lines with apparently normal characteristics were easily established from patients with malignant melanoma.

Tumor cell vs lymphocyte *in vitro* interactions are best studied in an autochthonous system. An allogeneic system adds the confusion of transplantation isoantigen reactions and possibly other unknown antigenic differences.

The semimicroplate assay described requires large numbers of tumor and lymphoid cells compared to other assays and is, therefore, useful only for studying

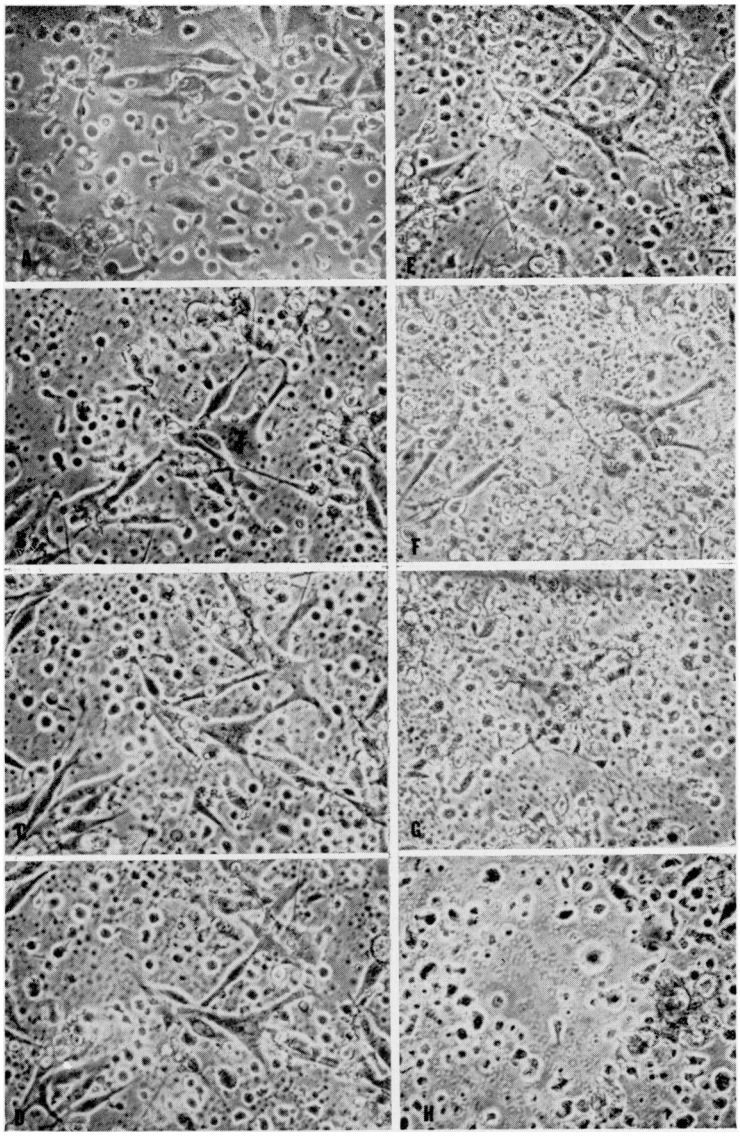
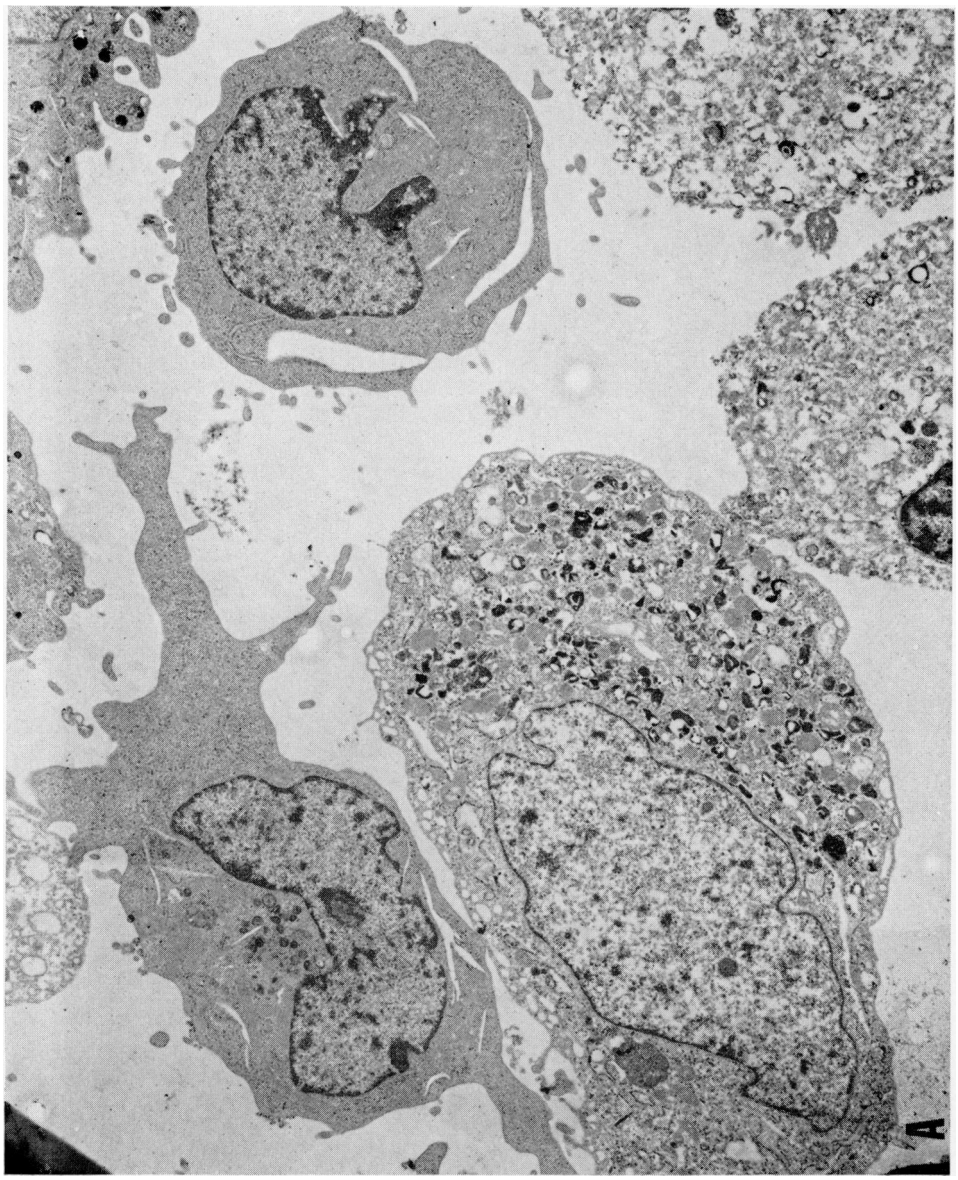


FIG. 3. This is a collage of photomicrographs representing a time lapse film made as described in the Methods. During the course of the movie, the lymphoid cells (RPMI 7711) changed from primitive "blast" forms to mature "aggressor" cells. Motility was very obvious. Some of them atached to the target cells (RPMI M7821, T) and caused them to round up and even break up, presumably killing them. A, B, C, D, E, F, G, and H represent 0, 11, 23, 31, 40, 45, 50 and 70 hr, respectively. The only survivors appeared to be lymphoid in nature.

cultured cell interactions. It is, however, more versatile than most assays because the kinetics of the reaction can be conveniently and quantitatively followed.

Giemsa stain is a mixture of azure blue II and eosin. Although the biochemical mechanism of staining is not known, Giemsa appears to stain nucleic acid primarily. Since the stain can be extracted from cells by glycine–HCl buffer, it provides a unique method for determining the number of attached cells, regardless of lack



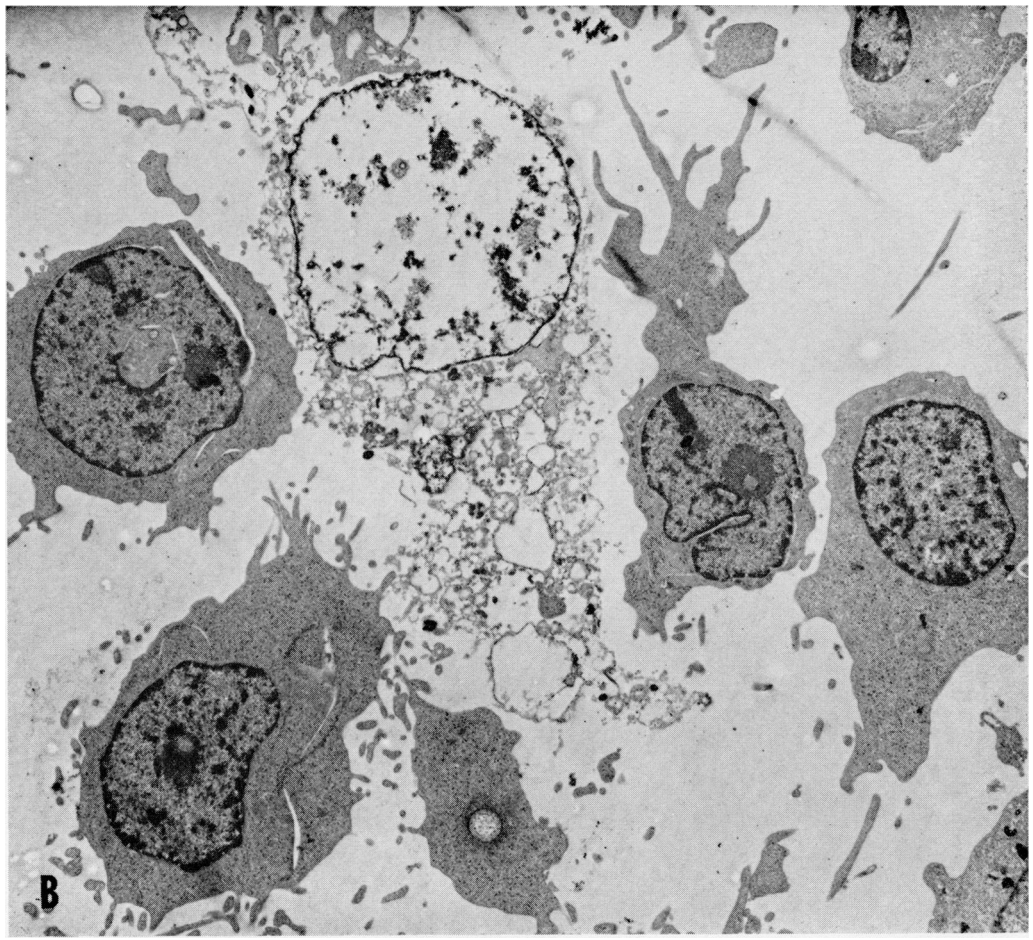


FIG. 4. (A) After 30 hr of interaction, this culture was fixed for electron microscopy. A melanin-containing target cell (RPMI M7821) has a lymphoid cell (RPMI 7711) attached to it. Osmium, uranyl acetate, lead citrate. × 7,000. (B) After 60 hr of interaction, the majority of melanin-containing cells appear to be disintegrating. Osmium, uranyl acetate, lead citrate. × 7,000. Neither emperipolesis nor phagocytosis of melanin granules was observed.

of surface contact inhibition. Giemsa stain is used because the background after destaining is very low compared to other more specific stains. This new system is convenient for several reasons, viz., the autogenic cells are continuously available in large amounts. There are no stroma cells or humoral factors interferring with reactions. Since the cells grow on the same medium, they can be mixed together in culture.

As was shown, some lymphoid cell lines do not contain many killer cells. Preliminary experiments indicate that these non-killer cells can be activated by preincubation with the target-tumor cells and that the reaction can be "blocked" by some, but not all, autologous sera. Active lymphocytes were quite motile and able to attach to tumor cells, kill them, dissociate themselves, and reattach and kill additional tumor cells. Attachment is evidently required since the lymphoid cells cannot be washed off the tumor cells with fresh medium after 48 hr. The survivors of the interaction are lymphoid cells.

Electron microscopic examination of the cell-cell reactions revealed disintegrating melanin-containing cells with lymphocytes attached.

In comparable experiments, lymphocytes neither killed nor inhibited the growth of autochthonous fibroblast cells.

SUMMARY

The establishment and characterization of thirty malignant melanoma cell lines and their maintenance under optimal growth conditions are described. One-hundred-sixteen lymphoid cell lines have also been established from melanoma patients. Preliminary evidence is given to support the claim that some lymphoid cell lines retain the ability to recognize and kill autochthonous malignant melanoma target cells after several months in culture. The interactions of such mixtures were evaluated by a new semimicro plate assay, by time-lapse, phase-contrast cinemicrography, and by electron microscopy.

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