Cell Tissue Res (1981) 214:431–441

Cell and Tissue Research
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Thymic Microenvironment and Cultures Derived from Mouse Thymic Explants

A Morphological Study

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Summary. Cultures derived from thymus fragments of embryonic (18–19 day old), newborn or one month old C57BL mice have been characterized functionally (phagocytic and nonspecific esterase activities) and morphologically by means of light, scanning (SEM) and transmission (TEM) electron microscopy. The observations show the heterogeneity of the cell populations composing the monolayers. After a few days incubation macrophages appear as the predominating cell type, while epithelial cells usually constitute no more than 30% of the cells. Experiments designed to determine the fate of lymphocytes adhering to the monolayers lead us to believe (on the basis of SEM morphometric analysis) that the survival of lymphocytes attached either to thymic macrophages or to epithelial cells is improved during the first days of coculture. This survival enhancement does not, however, appear to be a specific inductive effect since a similar survival increase is found when lymphocytes adhere to non-thymic cells. In contrast with the monolayer, the explant provides a three-dimensional culture system able to preserve intact thymic microenvironmental conditions since numerous lymphocytes are found even in five week old cultures which were not overlaid with thymocytes or spleen cells.

Key words: Thymus – Microenvironment – Culture – Epithelial cells – Macrophages.

It is generally admitted that the maturation of T lymphocytes requires interactions (cell to cell contact or humoral mediator factors) with other non-lymphoid cells. In this respect, the use of in vitro pure monolayer cultures of non-lymphoid cells could provide a simple approach to the analysis, under controlled conditions, of the interactions between lymphocytes and the non-lymphoid elements.

Most of the non-lymphoid cells of the normal thymus are of epithelial nature. With their long branching processes they constitute most of the thymus supporting

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0302-766X/81/0214/0431/$02.20

framework. Besides epithelial cells there are fibroblasts which are generally associated with the capsule and the blood vessels; macrophages are also usually found within this framework (Kostowiecki 1963; Hoefsmit and Gerver 1975; Bearman et al. 1978).

Thymus derived cultures are often considered as composed of epithelial cells. Although the presence of fibroblasts and macrophages was previously recorded in monolayer culture derived from the mouse (Mosier and Pierce 1972; Jordan et al. 1979) or human thymus (Pyke and Gelfand 1974; Hensen et al. 1978), most authors do not take into consideration the heterogeneity of such cultures.

In order to investigate the ability of primary cultures derived from mouse thymic explants to maintain an original thymic microenvironment, it was necessary to characterize the cellular composition of such cultures on the basis of morphological and functional criteria. Attempts were made in order to estimate the possible effect of such cultures on lymphocyte survival.

Materials and Methods

Culture of Thymus Explants. Embryonic (18–19 day old), newborn or one month old C57BL mice were sacrificed by cervical dislocation; the thymuses were removed aseptically and put in sterile phosphate buffered saline (PBS). They were minced into small pieces with scissors and explanted to culture dishes (Costar 3506). The fragments were initially cultured in Waymouth's medium supplemented with 5% foetal calf serum, $2\,mM \cdot ml^{-1}$ L-glutamine, $150\,U \cdot ml^{-1}$ penicillin and $0.075\,mg \cdot ml^{-1}$ streptomycin. The cultures were placed at 37°C in a 5% CO_2 humidified atmosphere for 48h. Thereafter, Waymouth's medium was replaced with MEM Eagle medium supplemented with 10% foetal calf serum, $2\,mM \cdot ml^{-1}$ glutamine, $150\,U \cdot ml^{-1}$ penicillin and $0.075\,mg \cdot ml^{-1}$ streptomycin. The cultures were observed regularly by using an inverted phase microscope and the medium was changed every 2–3 days.

Embryo Fibroblast Cultures. Twenty day old foetuses were removed aseptically and placed in sterile PBS. They were cut with scissors into small fragments and explanted to culture dishes (Costar 3506). All the culture conditions were similar to those described for the thymic explant cultures.

Cultivation of Thymocytes and Spleen Lymphocytes on Thymic Explant Cultures. Thymuses or spleens were removed aseptically, put in PBS, and minced into small pieces with scissors. The suspension was filtered through tissue nylon Blutex (100 µm meshes). The cells were washed by centrifugation and resuspended in MEM Eagle's medium (10^6 cells $\cdot\, ml^{-1}$). Two ml of these suspensions were added to the thymic explants cultures. At the end of the cultivation period, non-adherent thymocytes and spleen lymphocytes were recovered by gentle pipetting. These cells were washed by centrifugation and their viability was determined with the SEM (see results). Similar studies were performed after incubation of thymocytes and spleen lymphocytes with embryonic fibroblasts and in culture dishes in the absence of any other tissue.

Latex Particle Phagocytosis and Non-Specific Esterase Activity. Cultures were incubated at 37°C for 1 h with 2 ml of a 0.091 µm latex particle suspension ($10^6 \cdot ml^{-1}$ particles). Thereafter, they were washed with PBS and fixed for examination with a transmission electron microscope. Similar cultures were treated by the α-naphtylacetate method in order to detect the cells with non-specific esterase activity (Pearse 1972).

Scanning Electron Microscopy (SEM). Slides supporting either monolayers or cocultures at various delays or control suspensions sedimented on polylysine-coated slides were immersed for 15–30 min at room temperature in a 2.5% glutaraldehyde phosphate buffered solution. After osmication in 1% OsO_4 and dehydration in graded ethanol and ethanol-amylacetate series, the samples were transferred to a critical point drying apparatus using CO_2. The slides were attached to stubs, coated with gold/palladium

by sputtering and examined in an ETEC microscope with an accelerating voltage of 20 KV and a tilt angle of 45°.

The survival rates of cocultivated lymphocytes was evaluated with the SEM by counting the number of alive and degenerated lymphocytes among those in close contact with the cell substrate. By using a semi-automatic Leitz A.S.M. image analysis system, the average number of lymphocytes in contact with epithelial cells or epithelial macrophages is expressed per surface unit of the latter cells.

Transmission Electron Microscope (TEM). Cultures were fixed in situ with a phosphate buffered 2.5% glutaraldehyde solution (pH 7.4). After osmication and dehydration in graded ethanol solutions, the samples were embedded in Epon 812. Serial perpendicularly-oriented thin sections of the monolayers were gathered on formvar coated grids, contrasted with uranyl acetate and Pb citrate and examined in a Siemens 101 electron microscope.

Results

Cell Composition of the Monolayers. Under our experimental conditions, explants stick to the plastic Petri dishes after 1 or 2 days of cultivation. Later on, various cell types start growing around the explants and eventually constitute a monolayer.

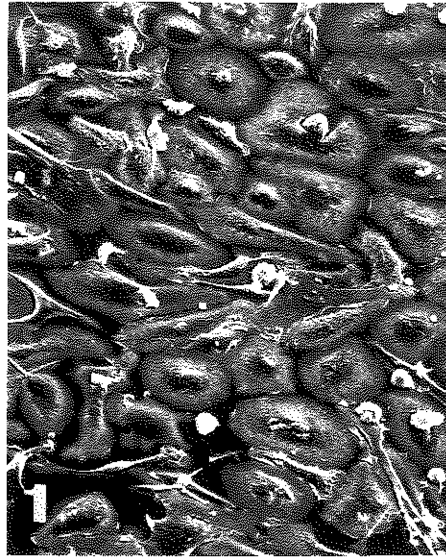
Approximately 10 days (embryonic thymuses) to 15 days (newborn and one month old thymuses) of culture are necessary to obtain confluent monolayers. They are composed of at least 3 morphologically distinct cell types: fibroblasts, macrophages and epithelial cells. The frequency of each type varies according to the duration of cultivation; there are also differences from one culture to another.

Macrophages are a constant component of the monolayers. Numeration of the cells positive for non-specific esterase reveals that macrophages may constitute up to 80% of the cell population. They may have a globular shape with dendritic processes but they often show also an epithelioid appearance forming, in some instances, large mosaic-like arrangements (Fig. 1) which, under the phase contrast microscope, may be easily confused with a true epithelium. Dendritic as well as epithelioid forms actively phagocytose latex particles (Fig. 5).

Epithelial cells are either isolated or grouped in small islands (Figs. 2 and 4); their nucleus generally contains two or more nucleoli. They do not show non-specific esterase activity and have no phagocytic capacities. Epithelial cells occasionally exhibit sparce tonofilaments and membrane junctional complexes (Fig. 6). Around the explant they may constitute larger areas of cells getting in close contact with each other (Fig. 3). In these areas, typical tonofilament bundles and conspicuous desmosomes are regularly observed (Fig. 7). Epithelial cells may exceptionally represent up to 60% of the confluent monolayers. On average, however, they constitute only 20 to 30% of the non-lymphoid cells; this proportion decreases with the age of the culture.

In contrast with the two former cell types, fibroblasts look like either individual elongated overgrowing cells (Fig. 1) or bundle-like structures running at the periphery of macrophage and epithelial areas (Fig. 4).

Survival of Thymocytes and Spleen Lymphocytes in Coculture with Thymic Derived Monolayers. The cultivation of spleen cells or thymocytes with thymus cultures does not improve the survival of the cells remaining in suspension as shown by the Trypan blue exclusion test. For example, under our experimental conditions, the percentage of thymocytes remaining alive is approximatively 90% on day 1, 55%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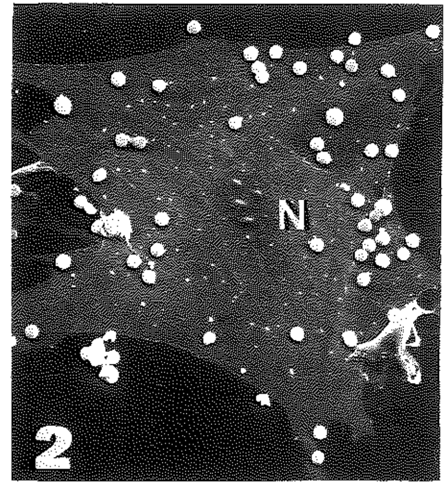
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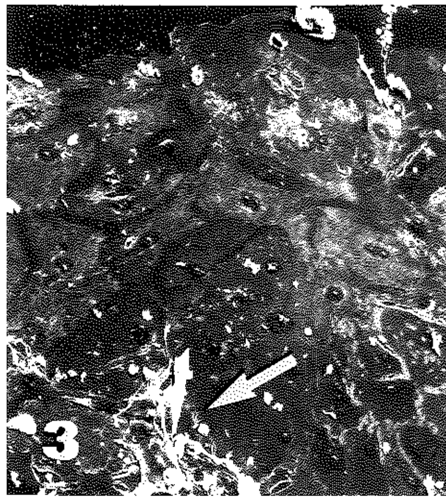
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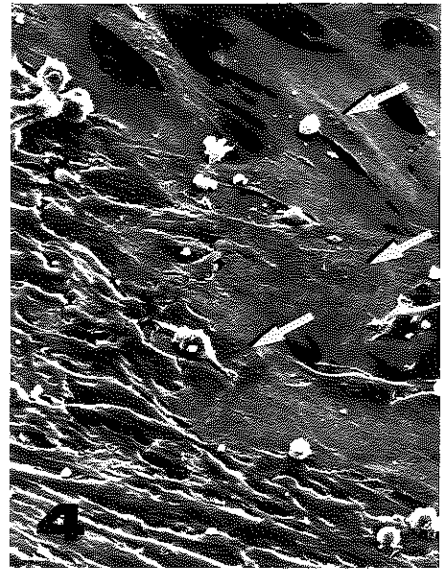
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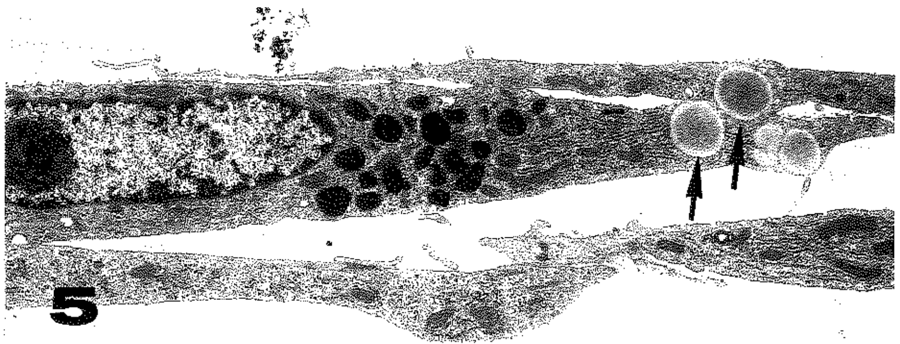
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on day 2, 20% on day 3 and less than 5% on day 7. After the removal of the culture medium containing the "floating" cells, a certain number of lymphocytes remains closely attached to the monolayer. Is it possible that lymphocytes in close contact with epithelial cells or macrophages survive longer than those in suspension?

The SEM allows an accurate analysis of this situation since degenerating lymphocytes exhibit typical membrane structure alterations, such as a decrease of microvilli and the appearance of membrane microperforations.

The variation of the total number and the survival rates of lymphocytes adhering to epithelial cells and macrophages at various times of incubation are reported in Figs. 9 and 10. It may be seen that the number of live lymphocytes decreases rapidly in the first few days of culture, whether they are in contact with epithelial cells or with macrophages (Fig. 9). On day 7, this number reaches less than 5% of the starting cell population. On day 10, living lymphocytes are no longer found adhering to the monolayer. These results were compared with those obtained with thymocyte or spleen cells either cultured in Petri dishes in the absence of any feeder layer or kept in suspension (Fig. 10). During the first 48 h, the survival of lymphocytes adhering to the thymus monolayer is 100 to 200% higher than the controls. Later on, the difference of survival is reduced. However, this transient survival improvement is not restricted to a specific contact with thymus cells since comparable survival rates are observed when embryo fibroblast monolayers are overlaid with spleen lymphocytes.

Thus, lymphocytes of overlaid thymus monolayers (as well as lymphocytes of primary cultures) degenerate during the first days of culture. However, examination of thin sections perpendicularly oriented to the monolayers, reveals the persistence of normal looking lymphocytes trapped beneath the monolayer while lymphocytes adhering to its surface are degenerating (Fig. 8). That these "surviving" lymphocytes may originate from the explant itself is demonstrated by their presence in 30 day old cultures which were not overlaid with thymocytes or spleen cells.

Morphological Particularities of Thymic Explants in Relation to Lymphocyte Survival. In 30 day old primary cultures, the surface is composed by elongated contiguous cells with flattened nuclei and few cytoplasmic inclusions (Fig. 11). The

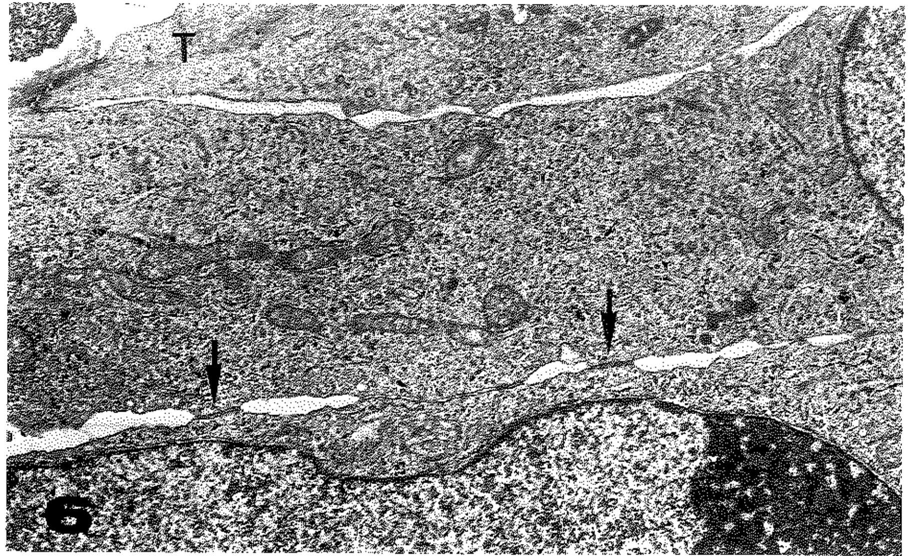
Fig. 1. SEM micrograph showing macrophages associated in an epithelium-like arrangement in a 19 day old thymus derived monolayer. Note the presence of typical dendritic and small globular macrophages as well as sparce overgrowing fibroblasts. × 250

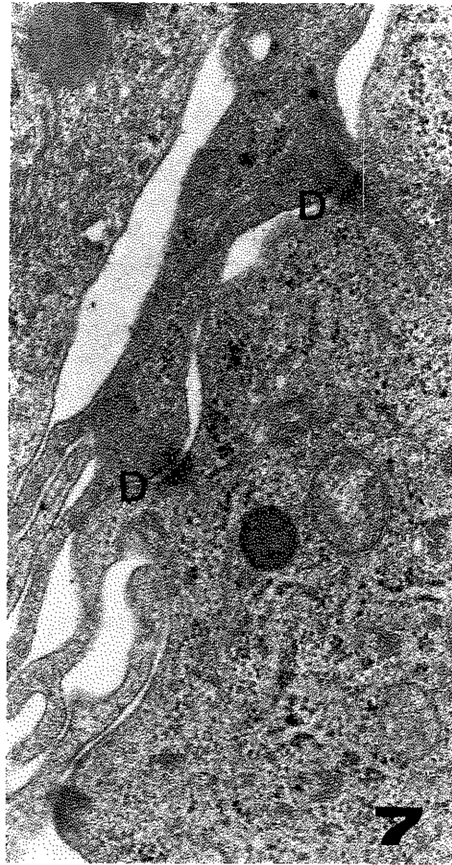
Fig. 2. Isolated epithelial cell from a 18 day old monolayer 24 h after overlying with thymocytes. The nucleus (*N*) contains 4 nucleoli. SEM. × 500

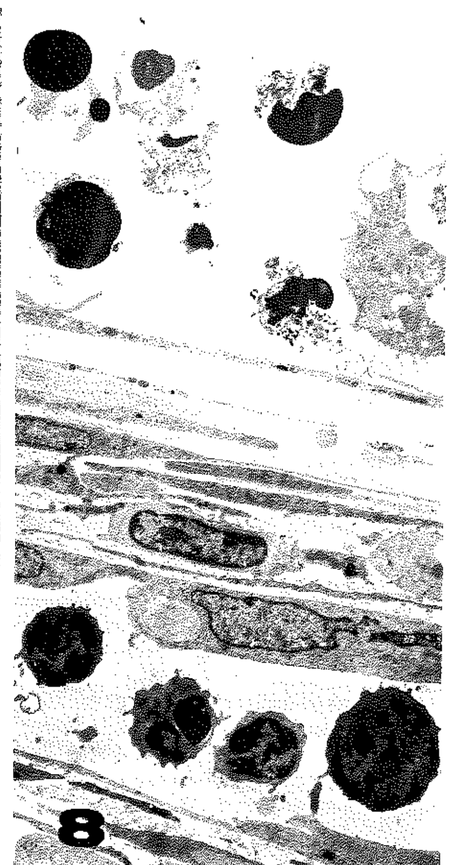
Fig. 3. Part of a 20 day old culture showing the explant area (*arrow*) surrounded by a continuous epithelial cell monolayer. SEM. × 125

Fig. 4. SEM view of fibroblast bundles concentrically running around an epithelial cell island. The *arrows* indicate the multinucleolated nuclei of epithelial cells. × 250

Fig. 5. Fine section of an epithelioid-like macrophage with ingested latex particles (*arrows*); 19 day old culture. TEM. × 7,550







Figs. 6–7. Organization of epithelial cells in monolayer (**Fig. 6**) and explant (**Fig. 7**) areas, respectively; compare the faint junctional membrane complexes (*arrows*) of **Fig. 6** with true desmosomes (*D*) of **Fig. 7.** *T* tonofilament bundles; 20 day old culture. TEM. (**Fig. 6,** ×6,400; **Fig. 7,** ×30,100)

Fig. 8. Perpendicularly-oriented section of a 21 day old culture close to an original explant showing four "living" lymphocytes beneath flat cells. Lymphocytes in the outer medium are degenerated. TEM. ×2,500

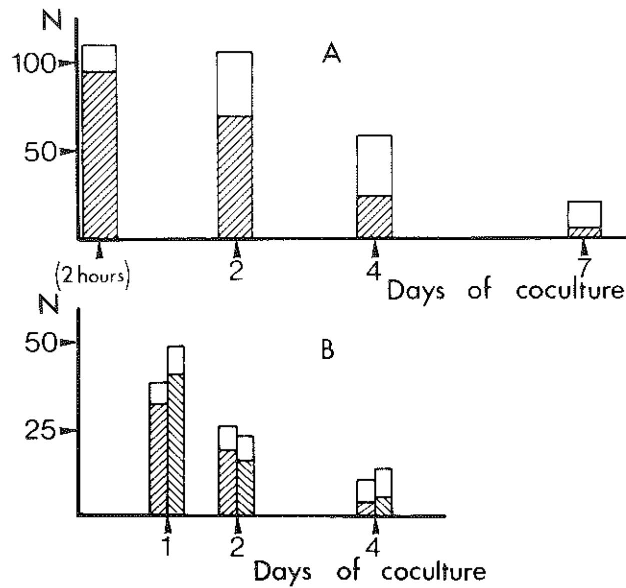


Fig. 9. Variations of the total number (N) of lymphocytes adhering to epithelial cells (▨) and macrophages (▧) at various times of incubation. The open part of columns indicates the fraction of degenerated lymphocytes. The results are expressed as the number of lymphocytes of spleen (A) or thymus (B) origin per $10^{-2}\,\mathrm{mm}^2$

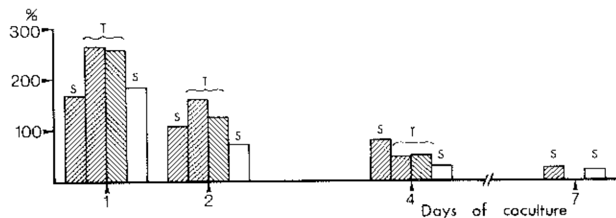
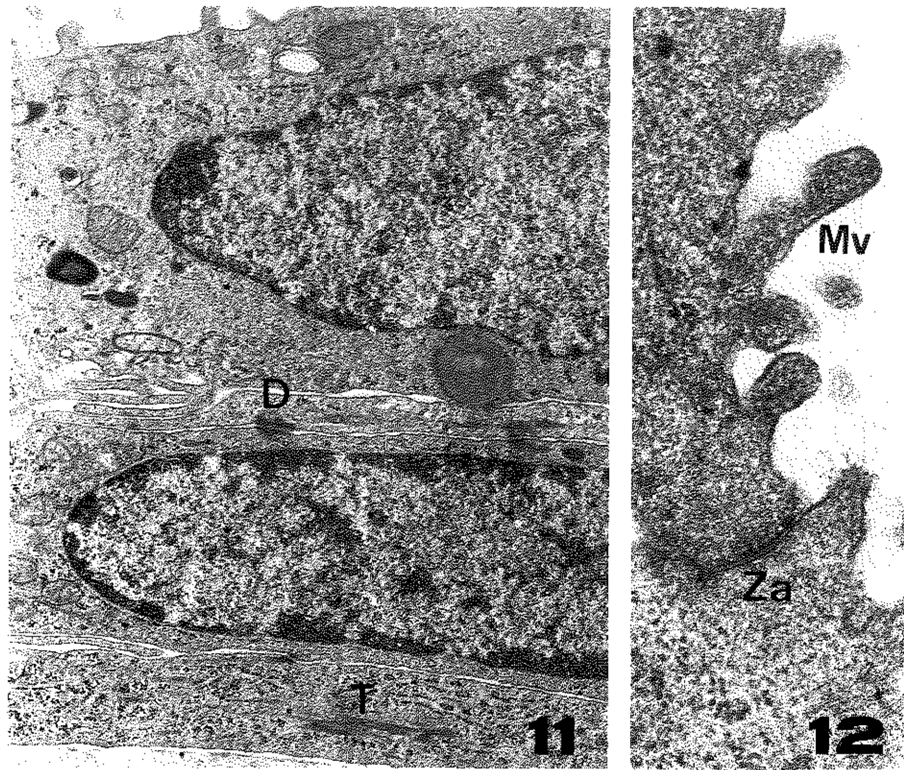
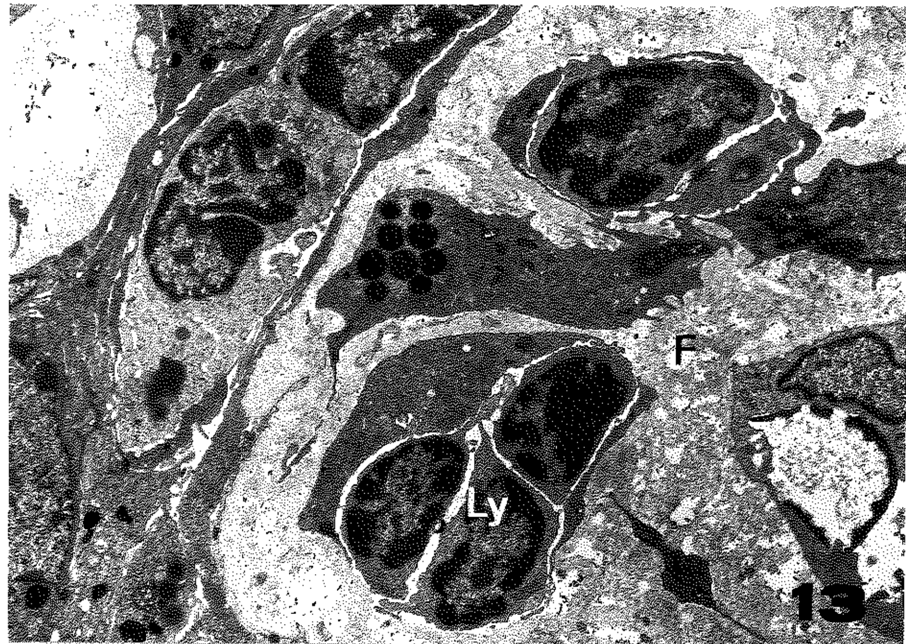


Fig. 10. Survival increase of spleen (S) or thymus (T) lymphocytes adherent to cultured non-thymic embryo fibroblasts (▭) or to epithelial cells (▨) and macrophages (▧) of thymus derived monolayers. Values are expressed in % and calculated from the relation $\frac{\mathrm{Tc} - \mathrm{TE}}{\mathrm{TE}} \cdot 100$ where Tc and TE represent the lymphocyte mortality rates in controls and in cell cultures respectively

plasma membranes form short microvilli directed forwards in the culture medium. Laterally, these membranes are sinuous and imbricated with each other, taking sometimes the appearance of a *zonula adherens* (Fig. 12). Typical desmosomes and tonofilament bundles are regularly observed (Fig. 11). In contrast with this typical epithelial organization, the non-lymphoid elements of the deeper part of the explant (Fig. 13) are stellate or spindle shaped cells. They are epithelial cells and macrophages. Usually, they build up a loose framework filled with clusters of lymphocytes. This framework itself is transsected by fine fibrous material and collagen fibers. The lymphocytes found in this framework have a normal appearance. Serial sections made at the periphery of the explants also show





Figs. 11–12. Typical feature of cells constituting the superficial area of a cultured explant (20 day old). *D* desmosomes; *T* tonofilament bundles; *Mv* microvilli; *Za* zonula adhaerens-like formation. TEM. (**Fig. 11,** ×14,700; **Fig. 12,** ×42,000)

Fig. 13. Deep part of a 20 day old cultured explant. Lymphocytes, grouped in small clusters (*Ly*), are separated from the intercellular fibrous material (*F*) by cytoplasmic expansion of stellate and spindle-like cells. TEM. ×4,800

numerous lymphocytes beneath the flattened cells extending outward from the explant edge.

Discussion

Our results show the great heterogeneity of the cell population composing the monolayers obtained by cultivating thymus explants. After a few days incubation, macrophages appear as the predominating cell type, while epithelial cells usually constitute no more than 30 % of the cells. Except for Jordan and Crouse (1979), who recently described the morphological aspects and the growth pattern in a system similar to ours, the observations we have reported present some discrepancies with those of other investigators. Though the presence of fibroblasts and/or macrophages has occasionnally been mentioned as contamination material (Raedler et al. 1978; Mosier and Pierce 1972; Kruysbeek et al. 1978), thymus derived monolayers are generally considered as thymic epithelium. It must be noted that the mosaic-like pattern of epithelioid macrophages can easily be confused with a true epithelium in light microscopy. This is probably the main reason why the cell heterogeneity of such systems had been ignored.

The relative scarcity of tonofilaments and desmosome-like junctions reported in epithelial cells of mouse thymic monolayers (Jordan and Crouse 1979; the present observations) seem to be particular to this species by comparison, for example, with rat (Kruysbeek et al. 1977) or human (Hensen et al. 1978) cultured thymus.

Up to now, experiments designed to determine whether the contact with thymic derived monolayers extends the in vitro life span of lymphocytes have led to negative conclusions. However, these observations were based on direct numeration of live and degenerated lymphocyte populations removed from the culture medium. The results we obtained by using the SEM in order to study the fate of lymphocytes adhering to the monolayer indicate that the survival of lymphocytes attached either to thymic macrophages or epithelial cells, is improved during the first days of coculture. However, a similar survival increase is found when lymphocytes adhere to non-thymic cells. This suggests that the survival enhancement may be related to a general non-specific "contact" effect ("trophic" factor?).

It has been shown in vitro that epithelial cells either in mixed or in pure secondary monolayer culture (Boniver et al. submitted; Wekerle et al. 1973; Waksal et al. 1975; Sato et al. 1976; Kruysbeek and Astaldi 1979) as well as macrophages (Lopez et al. 1977; Beller and Unanue 1978) are involved in the differentiation of precursor cells into T lymphocytes. The present data clearly demonstrate however that by using monolayers of confluent cultures derived from mouse thymus fragments, it is impossible to keep lymphocytes in long term cultures.

By contrast, our explant culture system seems to maintain a three-dimensional structure able to preserve intact thymic environmental conditions since numerous lymphocytes are regularly found even in five week old cultures. These observations agree with those of some authors (Mandel and Kennedy 1978; Robinson and Owen 1976; Juhlin and Alm 1976) working with foetal thymus cultures in which an increase and a maturation of lymphocytes were observed during the 15 first days of cultivation. Furthermore, the viable lymphocyte aggregates enclosed by stromal

cells which we often found in 20–30 day old explants display a morphological feature comparable to the thymus "nurse cells" organization recently described by Wekerle and Ketelsen (1980). As suggested by these authors, these cells of epithelial nature provide microenvironmental requirements necessary for lymphocyte proliferation and differentiation. According to other workers, cultivation of thymic fragments from *neonatal* or *adult* mice results in a progressive depletion of lymphoid cells (Schulte-Wisserman et al. 1978, 1979; Hong et al. 1979), reaching a complete depletion of lymphocytes from the tissue (generally considered as epithelial!) after 10, at the most 20, days of culture.

Studies are in progress in order to determine whether lymphoid populations of our cultured explants are exclusively surviving original lymphocytes or result from an *in vitro* cell proliferation. Also, our explant culture system provides a model which is presently used to study the effect of specific agents (such as Mycostatin: Boniver et al. submitted) known to alter cell function and to investigate microenvironmental factors involved in the coordination of lymphocyte maturation.

Acknowledgements. We wish to acknowledge the technical assistance of Cl. Dumoulin, N. Gonda, R. Latet and J. Renson for histochemistry and electron microscopy, G. Righetti for iconography and A.M. Charlier for typing the manuscript

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Accepted October 12, 1980