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The differentiation of murine thymocytes *in vivo* and *in vitro**

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Summary. The differentiation of lymphoid cells in the early foetal mouse thymus was studied *in vivo* and in organ culture. The lymphoid precursors found in the 13 and 14 day foetal thymus constituted about 50% of the total thymus cell population. These T precursors were large blast-like cells which already expressed the Thy-1 antigen but were mostly TL negative. Both *in vivo* and *in vitro* the blasts were rapidly replaced by a population of typical small lymphocytes which were strongly Thy-1 and TL positive but Ig negative.

In organ cultures grown under optimal conditions, large bimodal increases in cell numbers occurred. An initial population of about 2×10^4 T precursors per thymus lobe gave rise to nearly 10^6 predominantly Thy-1 and TL positive small lymphocytes by by the 6–8th day *in vitro*. After this time, lymphocyte numbers decreased until about the 10th day when they again increased to form a second peak of small lymphocytes on the 12–13th day. These cells were also predominantly Thy-1 positive but the majority were now TL negative.

No Ig positive B lymphocytes were detected either by immunofluorescence or by autoradiography using polyvalent anti-MIg sera and no plasma cells were detected by electronmicroscopy At all times however, minor subpopulations of Thy-1 negative small cells were present. The production of small lymphocytes during the 1st week of culture was critically dependent on culture conditions and particularly on the batch of FCS used. The population developing during the 2nd week required less stringent conditions and was less dependent on FCS. The culture systems described should prove useful in the study of T-lymphocyte differentiation.

INTRODUCTION

It is now generally accepted that T lymphocytes are a highly heterogeneous group of cells consisting of a number of functionally and antigenically distinct subsets. Most T cells are morphologically similar and cannot be distinguished from one another by appearance in conventionally stained histological sections, in stained smears, or even with any certainty in the electron microscope but they can be readily separated functionally and by the analysis of their cell surface properties (Simpson & Beverley, 1977a, Simpson & Beverley, 1977b). However, the precise developmental relationships between the various subsets are still poorly understood. There are two major schools of thought regarding the differentiation of T cells within the thymus. One belief is that the T-cell precursors entering the thymus give rise to the cortical population, most of which die *in situ*, while some enter the thymus medulla and then

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migrate to the peripheral lymphoid tissues (Weissman, 1967, 1973). The other, contrasting, belief suggests that there are at least two major subsets of T lymphocytes which do not bear a product-precursor relationship to each other and which arise independently in the thymus (Schlesinger, 1972; Shortman & Jackson, 1974; Hopper & Shortman, 1976).

There are, however, serious difficulties in studying intrathymic T-cell differentiation *in vivo* since cell migration, both into and out of the thymus, cannot be controlled or even readily monitored and stress effects due to the handling of experimental animals can give rise to massive thymus atrophy, particularly of its cortical regions. Studies using dispersed thymus populations are also unsatisfactory for long term cultures since dispersed T cells rapidly die *in vitro* even in the presence of underlayers of thymic epithelial cells (Mosier & Pierce, 1974).

More physiological *in vitro* methods for T-cell development do exist and have been used by a number of workers to study thymus differentiation. These methods are all based on modifications of an organ culture system, described initially by Auerbach (1960), and used subsequently by many workers (Moore & Owen, 1967; Owen & Ritter, 1969; Mandel & Russell, 1971; Juhlin & Alm, 1976; Tufveson, Juhlin, Steinbeck & Alm, 1976; Robinson & Owen, 1976). In this paper we describe culture conditions which optimize the development of the foetal mouse thymus *in vitro* and compare the development of 14 day foetal thymus in closed organ culture systems with its development in the intact foetus. We suggest that, when optimal culture conditions are used, it is possible to study T-cell development in a totally closed and controlled system under conditions which appear to approximate those present in the developing foetus. Furthermore we show that under such conditions two subpopulations of Thy-1 positive small lymphocytes develop from the small precursor population of large blast like cells present in the explanted 14 day foetal thymus (Mandel, 1970; Mandel & Russell, 1971; Moore & Owen, 1967).

MATERIALS AND METHODS

Animals

Mice used for most experiments were inbred CBA/H/WEHI and (CBA × C57Bl/6)F_1 animals bred at The Walter and Eliza Hall Institute and raised either under conventional conditions, or more recently, under specific pathogen-free conditions. For some experiments BALB/c and C57Bl/6 mice, and for the study of the expression of the TL antigen congenic A/J (TL^+) and ATL^- strains (obtained from Dr E. A. Boyse) were also used.

Intact thymus lobes dissected out of 13 or 14 day foetuses were used for organ cultures. The age of gestation was determined by vaginal plugging; day 0 of gestation being taken as the day on which a vaginal plug was found. The degree of development for a given gestational age varies with strain and 14 day foetuses of CBA, BALB/c, A/J and ATL^-, and 13 day foetuses of C57Bl/6 and (CBA × C57Bl)F_1 mice were used as these were developmentally comparable. However, since litters of the same calculated gestational age showed some variation in development, possibly reflecting variations in actual time of fertilization, the litters used for any one experiment were selected so as to minimize such variation.

For the assessment of normal thymic development, intact thymus lobes were dissected from a series of CBA and (CBA × C57Bl)F_1 foetuses of dated gestational age ranging from 13–19 days, and from neonatal and young adult mice. Gestational staging was further standardized by reference to the criteria described by Theiler (1972) for CBA mice.

Isolation of thymus lobes

The foetuses were removed aseptically and placed in sterile Eisen's Balanced Salt Solution (EBSS) containing 10–15% foetal calf serum (FCS, Commonwealth Serum Laboratories, Melbourne, Australia). The thoracic segments containing the heart, great vessels and trachea and the attached thymus lobes were isolated and placed in sterile EBSS-FCS and kept on ice until use. For any one experiment, 8–15 litters (40–100 foetuses) of identical strain and developmental stage were used.

Organ culture technique

Two major variations of organ culture were used. In our earlier studies we used individual plastic organ culture dishes (Falcon Plastics No, 3010). Each dish contained an insert of thin perspex in which were drilled two 3 mm holes. A piece of either Millipore (25 mμ 0·45 mμ pore size) or Nuclepore filter (General Electric, pore size 0·8 mμ) was glued over the holes and a single lobe was placed over each hole. Each

dish contained 1 ml of medium and the tissue was at the gas-medium interface. More recently we used 9 cm plastic petri dishes (Kayline Plastics South Australia) containing 15–16 ml of medium. The foetal thymus lobes were placed on to the surface of strips of sterile Millipore filter which rested on blocks of surgical Gelfoam (Upjohn C. Kalamazoo, Michigan, U.S.A.). The Gelfoam absorbed the medium, and the Millipore filters with their attached foetal lobes were always kept moist while remaining in the gas phase. In each 9 cm petri dish, between 25–30 lobes were arranged around the periphery of the Gelfoam-Millipore assembly. Media were usually changed every 3rd day in both culture techniques, but on occasions media were also changed at 2 or 4 day intervals with no apparent variation in cell growth providing other factors were kept constant.

Media and foetal calf serum

During the course of these studies three different media were compared. These were Eagles Basal Medium, Eagles Minimal Essential Medium and Dulbecco's Modified Eagles Medium (DME). Since DME consistently resulted in the greatest cell proliferation (all other conditions being equal) all studies reported here were performed with this medium.

The DME was always supplemented with 15% foetal calf serum (FCS). We found that different batches of FCS, obtained from a number of commercial sources, varied greatly in their ability to support cell growth, particularly in the 1st week of culture. Samples from a number of batches were tested in one experiment and the whole of the most suitable batch was bought and used for subsequent experiments. Sufficient FCS of a single selected batch was usually available for experiments for 12–18 months and no variation in its potency was observed during this time. The media also contained penicillin (100 μg/100 ml) and streptomycin (100 μg/100 ml) and 3·4 g/l sodium bicarbonate. The cultures were grown in a fully humidified incubator in 10% CO_2 in air at 37·5°.

Cell counts and cytologic assessment

Preparation of single cell suspensions by mechanical dispersion of young foetal thymuses of less than 16–17 days gestation and of cultured thymuses gave highly variable results, and particularly with the cultured tissue, variable and frequently poor cell viability. In addition a non-representative population was obtained since the non-lymphoid cells, which can form a significant proportion of the total cell population, were selectively killed during mechanical disruption of the organ. A gentler and a more reproducible method of cell dispersion was therefore necessary and incubation in trypsin (0·12–0·25%) with EDTA (0·02%) for 15–30 min at 37° in phosphate buffered saline (PBS) was found to be suitable (de Oca, Probat & Grubbs, 1971).

Cultured thymuses were harvested by gently pushing them off the supporting filter with the blunt edge of a cataract knife. This resulted in minimal cell loss as only a thin rim of the outgrowth of non-lymphoid cells remained attached to the filter and the bulk of the cultured lobe remained intact. The cultured thymuses and foetal thymus lobes were washed once in protein-free EBSS and were then placed in 1 ml of the enzyme solution. The trypsin solution was then removed and was replaced with EBSS-FCS to stop further enzyme action. The cells could then be readily dispersed into a single cell suspension by gentle aspiration with a Pasteur pipette. Reproducible and representative suspensions with a viability of over 95% as assessed by dye exclusion with either 1% trypan blue or 1% eosin were obtained. Cell counts were made in a haemocytometer from pooled cells from 8–12 lobes per sample and are expressed as the total cells per lobe. An impression of the cell types present could also be made in the haemocytometer and an estimate could be obtained of the ratio of lymphoid to non-lymphoid cells and of the size distribution of the lymphoid cells. Lymphoid cells were generally small and round with a sharp even border whereas the non-lymphoid cells were quite heterogeneous in appearance and were frequently large and irregular in outline.

For a more accurate assessment of the cytology of the dispersed cells, Giemsa stained smears were examined with an oil immersion lens. Non-lymphoid cells frequently disintegrated in the preparation of the smears making their precise identification impossible. Representative cell suspensions prepared from foetuses and from cultured thymuses of different ages were also fixed in diluted Karnovsky fixative (Karnovsky, 1965) and were processed for electron microscopy using standard procedures.

Analysis of cell surface characteristics

The presence of surface immunoglobulin (SIg) and of the Thy-1 and TL antigens, was assayed by direct

and indirect immunofluorescence respectively, using Ploem epi-illumination. A commercial polyvalent fluorescein-conjugated rabbit anti-mouse Ig (Fl-R-anti-M Ig, Beringwerke Ag. Marburg, Germany) and a batch of rabbit anti-mouse Ig prepared by Dr J. Goding of this Institute were used for direct IF. Each batch of antiserum was first titrated against normal adult spleen cells for anti-SIg activity, and the antiserum was finally used at a concentration which was obtained from the plateau of the resulting titration curve (generally 1 : 16 or 1 : 32). Anti-Thy-1 serum, obtained from Dr J. F. A. P. Miller, was produced as previously described (Miller & Sprent, 1971) and it too was first titrated against normal young adult CBA thymocytes with a 1 : 16 dilution of FI R-anti-MIg as the second layer. Prior to its use on foetal and cultured cells the antiserum was absorbed against an equal volume of 13-day CBA foetal liver cells to remove possible autoantibodies.

To assay for the presence of the TL antigen, mice of the TL^+ A/J strain were used. The anti-TL antiserum was a generous gift from Dr U. Hämmerling of the Memorial Sloan Kettering Cancer Center, N.Y. (Boyse, Old & Luell, 1963). Prior to its use this antiserum was tested on normal young adult A/J thymocytes (>90%+ve) and on young adult ATL^- thymocytes (<5%+ve). The second layer was again a polyvalent Fl-R anti MIg. The anti-TL serum was used at a final concentration of 1 : 8.

In all assays for either antigen, in both cultured and normal foetal thymus cells, controls using only Fl R anti-MIg were set up. These controls were consistently negative (<1% Ig^+ cells). Since it was important to rigorously exclude the presence of B cells for other experiments using this culture system, an attempt was made to introduce greater sensitivity for the detection of B cells by using ^{125}I-labelled R anti-MIg supplied by Ms. B. Pike of this Institute. Viable cells were labelled with this reagent and were processed for autoradiography.

Coulter counter analysis

Smears are essential for a study of cytology but for the determination of an accurate size distribution profile they are highly time consuming and open to serious sampling errors. A size distribution analysis was therefore performed on both normal and cultured thymus cell suspensions using a Model B Coulter Counter equipped with a particle size plotter. Cell suspensions, produced as described above, were resuspended in 'mouse tonicity' PBS (Shortman, Williams & Adams, 1972) at a concentration of 20–40,000 cells per ml and were analysed in the Coulter Counter. Multiple tracings (3 to 6) of each sample were made and from the resulting plot, curves were drawn and normalized to maximum peak height. Normal thymuses at different stages of gestation, and 13 or 14 day foetal thymuses cultured for varying times were then assayed.

RESULTS

Normal thymus development

The thymus anlage appears on about the 11th day of gestation and is soon colonized by immigrant stem cells (Moore & Owen, 1967). By the 13th day of gestation in CBA mice it is a minute but clearly defined structure consisting of two discrete oval lobes located one on either side of the lower trachea (Moore & Owen, 1967; Mandel, 1970). Histologically it is still largely undifferentiated but already contains numerous lymphoid precursor cells which can be distinguished from the epithelial and mesenchymal cells in Giemsa stained smears and sections (Moore & Owen, 1967; Owen & Raff, 1970) and by electron microscopy (Mandel, 1970).

In CBA mice each lobe contains about 20,000 cells on the 13th day of gestation, increasing to about 40,000 on day 14, and thereafter exponentially throughout gestation. In terms of both total cells and lymphocytes, at any one age BALB/c and A/J foetuses are approximately equivalent to CBA, but C57Bl/6 and $(CBA \times C57Bl)F_1$ are about 24 h more advanced (Fig. 1).

Smears of 13 and 14 day foetal cells show that approximately 50% of the cells are round and deeply basophilic with large nuclei containing prominent nucleoli (Fig. 2a). The remaining cells are of a similar size but contain a vacuolated and faintly basophilic cytoplasm, a more irregular nucleus and less prominent nucleoli. The edge of these cells is frequently irregular in contrast to the smooth outline of the lymphoid precursors and they tend to break up in smears. Ultrastructurally the lymphoid cells are also easily distinguished from the non-lymphoid cells both in suspensions and in intact organs. The lymphoid precursors contain numerous free polyribosomes but virtually no rough surfaced endoplasmic reticulum (RER), whereas the non-lymphoid cells are a heterogeneous group and show variable cytology with many cells containing bundles of cytoplasmic microfibrils, vacuoles and vesicles and

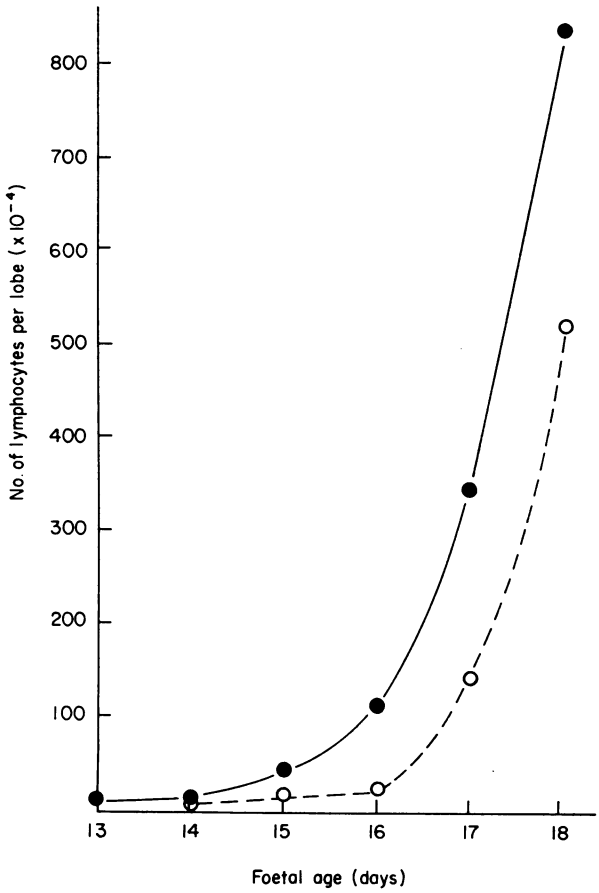

Figure 1. This figure shows the exponential increase in lymphocytes per thymus lobe from days 13 to 18 of gestation. Strains C57Bl/6 and (CBA × C57Bl/6)F_1 are indicated by (●), while strains CBA, A/J, ATL⁻ and BALB/c are indicated by (○). The proportion of non-lymphoid cells only makes a significant difference to the shape of the curves during days 13–15; after this time the lymphocytes constitute the majority of the cells of the developing thymus.

some RER (Mandel, 1970; Mandel & Russell, 1971). An interesting point is that, whereas the total cell number doubles between days 13 and 14, there are no obvious differences in the cytological appearances during this time and the proportion of lymphoid to non-lymphoid cells remains constant.

After day 14, an exponential increase in total cell number and a rapid and marked alteration in size distribution and in cell type occurs. The proportion of lymphoid cells increases rapidly (Table 1) and the distribution of lymphocytes also alters so that, shortly before birth, the majority are small and relatively few are large or medium in size (Table 1). The size distribution analysis obtained by Coulter Counter particle size analysis confirms this (Fig. 3). There is no significant change in size distribution between days 13 and 14. Thereafter there is a marked shift so that by day 18 the profile is practically identical with that produced by adult thymocytes, except for a shoulder of larger cells which is not seen in the normal adult profile (Fig. 3).

The surface characteristics of the trypsinized lymphoid precursors in 13 and 14 day foetuses were studied to determine whether they expressed Thy-1, TL or SIg. Trypsinized liver cells from the same foetuses and trypsinized adult spleen and thymus cells of the appropriate strain were used as controls. The controls showed that the antisera were active under the conditions used and that trypsinization, as used in these experiments, had a negligible effect

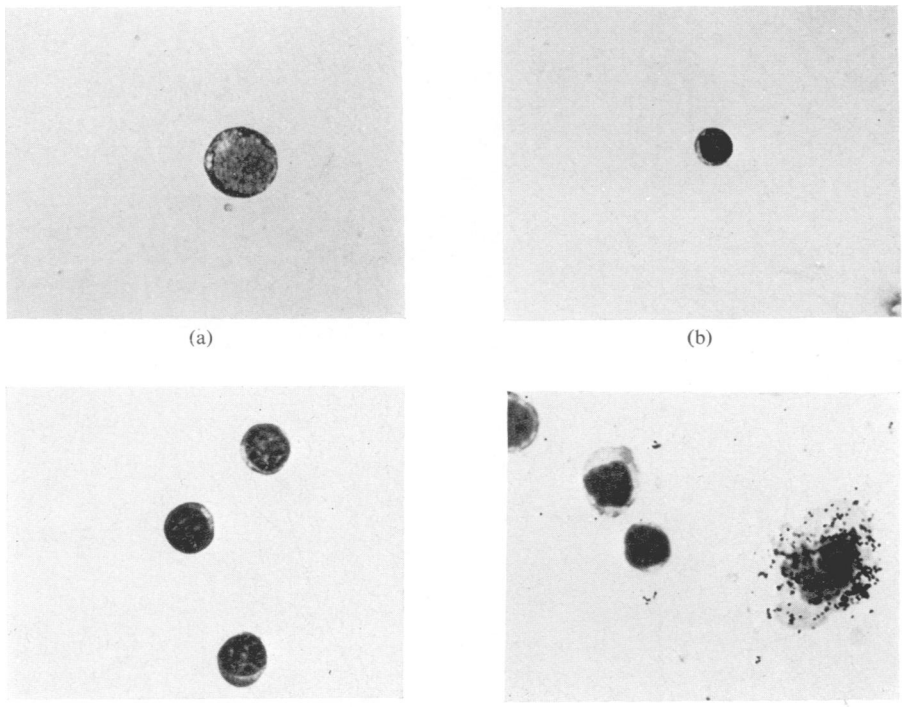

Figure. 2(a) A typical blast-like cell from a 14 day foetal CBA thymus suspension. Cells like this account for approximately 50% of the total cell population on days 13 and 14. Note the pale nucleus and copious cytoplasm. (× 1000, Giemsa stained smear). (b) A typical cell from a 7 day organ culture of 14 day foetal CBA thymus. Small lymphocytes comprise the majority of the lymphocyte population ($\simeq$ 80%) at this stage but a range of larger lymphoid cells is also present (e.g. see Fig. 2(d)). Cells of this type are also the commonest forms seen in late foetal, neonatal and young adult thymuses. (× 1000, Giemsa stain). (c) A group of small-medium lymphocytes from a 12 day organ culture. Compared to the cell illustrated in Fig. 2(b), these cells are larger and have a more open nucleus and more cytoplasm. Cells such as this comprise 60–80% of the lymphocyte population of 11–13 day cultures but constitute less than 20% of the lymphocytes seen on days 6–8. (× 1000, Giemsa stain). (d) An autoradiograph of a cell suspension prepared from a 6 day organ culture and exposed to polyvalent ^{125}I-rabbit anti-mouse immunoglobulin. The only labelled cell is large and has an irregular nucleus and extensive pale cytoplasm. Examination of stained smears not processed for autoradiography, and of electron microscope preparations, suggest that these cells are macrophages. Lymphocytes are unlabelled. (× 1000, Giemsa stain).

on the expression of either Thy-1 or TL (Scheid, Boyse, Carswell & Old, 1972) or indeed of SIg. No direct surface fluorescence with F1R anti-MIg was seen on 14 day foetal liver or thymus cells indicating the absence of readily demonstrable SIg. However under identical trypsinization conditions approximately 50% of adult spleen cells showed direct surface staining. No direct Ig staining cells were seen in foetal thymuses but a few brightly staining small round cells were seen in adult thymus reaching values of 2–4% in the atrophic thymuses of pregnant mice. Cytologically these SIg^+ adult thymus cells were identical in appearance and in staining intensity with SIg^+ spleen cells.

Over 95% of adult CBA thymus cells were Thy-1 positive by indirect IF (Table 2). When foetal liver absorbed anti-Thy-1 antiserum was used and was followed by F1-R anti-MIg, faint but distinct surface fluorescence was seen in about 60% of 14 day foetal CBA thymocytes but on no foetal liver cells. Capping of these cells occurred at room temperature. With increasing gestational age both the proportion of anti-Thy-1 stained cells and the intensity of the staining rapidly increased and by the 16th or 17th day of gestation the fluorescence was very bright and was practically identical in intensity to that seen on adult thymocytes. Thus the majority of recognizable lymphoid cells, even in very early foetal thymuses,

Table 1. Total and differential cell counts of the thymus of foetal CBA mice

Age*	Total cells per lobe ($\times 10^{-4}$)	Percentage†		Differential lymphocyte counts‡		
		Non-lymphoid	Lymphoid	Blasts	Med. ly	Small ly.
13	2	55	45	60	40	0
14	4	51	49	45	52	3
15	11	45	55	35	51	13
16	31	26	74	17	63	20
17	75	31	69	3	17	80
18	240	7	93	3	15	82
19	500	<5	>95	1	11	88

* Age of gestation was estimated as the days after finding a vaginal plug.

† The proportions of lymphoid to non-lymphoid cells were obtained from counts made of living cells in a haemocytometer since in the preparation of stained smears many (non-lymphoid?) cells were damaged and could not be identified. The non-lymphoid cells included macrophages, epithelial cells and fibroblasts.

‡ Differential counts were obtained from counts of 500–1000 cells examined under oil immersion in Giemsa stained smears.

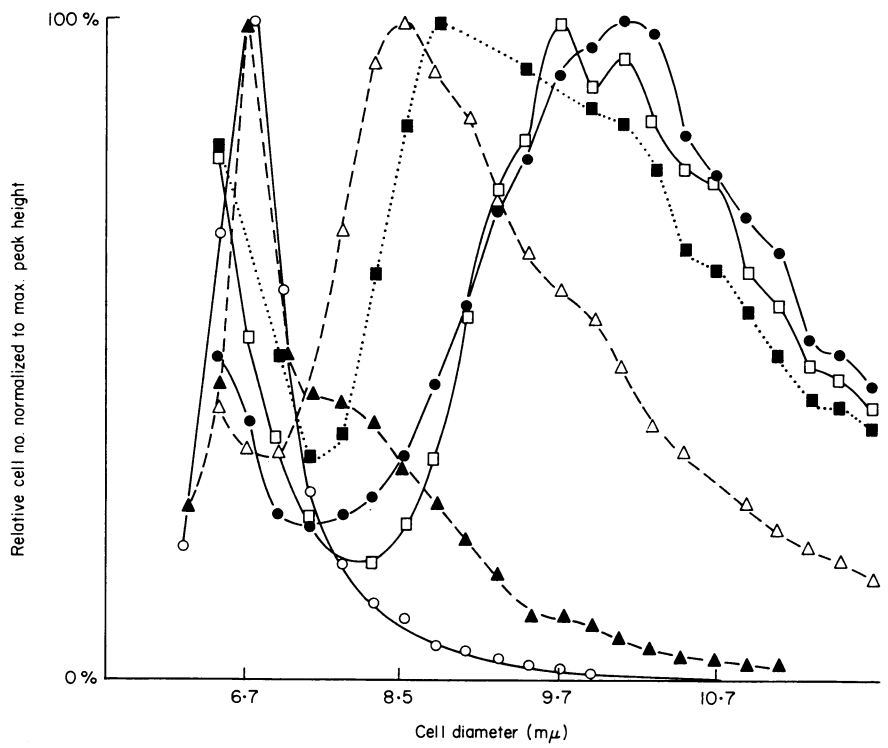

Figure 3. This figure shows the progressive decrease in cell diameter of foetal thymus cells taken at day 13 (□); day 14 (●); day 15 (■); day 16 (△); and day 18 (▲) and compares these with the size distribution profile of normal CBA adult thymocytes (○). The cells were passed through a Coulter Counter equipped with a particle size plotter. The curves are normalized to maximum peak height. The curve shows that little if any change occurs in the size distribution profile between days 13 and 14, then a rapid and progressive shift towards the left occurs so that by day 18 of gestation the cell profile resembles closely that produced by adult thymus cells. However a slight shoulder of larger cells is present in the 18 day foetal cells.

Table 2. The development of Thy-1 and TL antigens on the thymus cells of normal foetal, newborn and young adult mice. Antigens were detected by indirect immunofluorescence using polyvalent fluorescein-labelled rabbit anti-mouse Ig.

Age	Positive cells (%) TL*	Thy-1†	Ig†	Lymphocytes (%)
13	7·5	21	0	< 40
14	51	62	0	50
15	50	64	0	55–60
16	67	ND	0	70
17	ND	81	0	ND
18	91	95	0	90
19	90	95	0	95
NB†	95	> 95	0	97
Adult§	90	> 95	< 2	> 98

* A/J mice used in these studies.
† CBA mice; similar results obtained from A/J mice.
‡ < 24 h old.
§ Approximately 4–6 week old mice of either sex.

were Thy-1 positive, albeit weakly, when examined by indirect IF. This was in marked contrast to the total absence of Thy-1 positive cells in the foetal liver. A/J foetal thymus cells were examined for TL and about 50% were very faintly stained on days 14 and 15. The percentage of TL positive lymphocytes rapidly increased and reached values in excess of 90% by day 18 (Table 2). The positive cells also stained more brightly.

Thus the characteristic features of the 14 day foetal thymuses used for organ cultures were that they contained relatively few cells of which about 50% were large and lymphoid in appearance; the majority of these expressed the Thy-1 antigen, some were TL^+, albeit weakly, but none expressed free surface Ig.

Thymus development *in vitro*

The general appearance of the cultured foetal thymus lobes could be followed by examination at low magnification under a dissecting microscope. Initially the explanted lobes were oval, and at 14 days' gestation in CBA mice, measured about 0·2 mm × 0·1 mm. In most experiments very rapid growth occurred during the first few days *in vitro* and the cultures spread out to form flattened hemispheres. By day 7 each lobe was frequently 1–2 mm in diameter and a thin ragged outgrowth extended even further from the edge of the organ.

After the 7th day the cultures generally flattened and decreased in volume but they increased in thickness again toward the end of the 2nd week. A thin rim of flattened cells extended outward from the edge of the main culture. In the largest cultures, particularly in those grown in individual organ culture dishes, an opaque central area of necrotic tissue was sometimes present. When cultures were removed to prepare cell suspensions the rim of the out-growing cells was usually torn and remained on the filter. However the bulk of each cultured thymus remained intact.

Counts performed on trypsinized suspensions showed that rapid cell proliferation occurred during the first few days usually reaching peak values on days 6 or 7 (Fig. 4). The rate and amount of cell proliferation during this initial period was critically dependent on a number of factors. These were firstly, the type of medium used; consistently DME gave the greatest cell numbers, Eagles MEM was less effective while Eagles Basal medium, as used in previous studies (Mandel & Russell, 1971) was least effective. Secondly, the single most important factor determining cell growth was the batch of FCS used. Table 3 shows the results of a typical experiment in which a number of different batches of FCS were tested for their ability to support cell proliferation. It is apparent that great variation exists in the ability of different batches of FCS to support cell growth. The results shown in Table 3 were on thymuses grown in individual organ culture dishes but similar variation was observed with different batches of FCS when Gelfoam cultures were used. The third factor was the type of culture method used. While both individual cultures and Gelfoam assemblies in petri dishes gave similar maximum growth (approximately 10^6 cells per lobe on both days 7 and 12), the performance of the Gelfoam cultures was less variable (Table 4). This method was therefore chosen as the standard culture technique because, in addition to its greater reproducibility, it was also cheaper and faster since less manipulation was required during setting up and during media changes. The fourth factor which affected cell growth was variation in environmental conditions. Small fluctuations of either pH or temperature always resulted in poor cell growth during the first week but had less effect on older cultures. Under the best conditions, over 10^6 cells per lobe could be obtained on days 6–8 from the initial 20–40,000 cells present at the start of the culture. Growth in excess of this usually resulted in

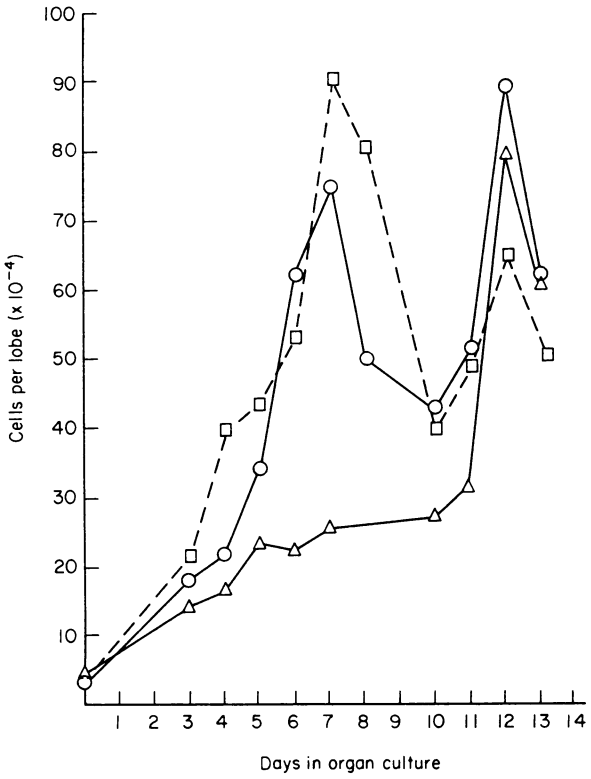

Figure 4. Total cell counts per lobe ($\times 10^4$) in three individual experiments in which 6–8 lobes were taken at daily intervals and trypsinized to produce a single cell suspension. The figure shows the variation which can occur between different cultures in relation to the actual and relative peak heights on days 7 and 12. In addition one culture (△) shows an absence of a first peak but still produced a large second peak on day 12. Each of these cultures was grown in organ culture dishes with two lobes per dish.

central necrosis of the lobes. However during trypsinization, necrotic cells were digested and were not seen as intact dead cells when cell counts were made, thus accounting for the high viability obtained.

After the 7th day cell numbers usually decreased until day 10–11 when a second increase occurred, usually reaching peak values on days 12–13. Thereafter total cell numbers again decreased. More than 10^6 cells were sometimes obtained at this stage (Fig. 4). The appearance of two peaks of cell growth was the usual mode of development observed in the majority of cultures. The timing of the peaks showed some variation; the first peak could occur between days 6 and 8 and the second between days 11–13 but peaks at days 7 and 12 were most commonly seen. In single experiments sampled daily, the heights of the two peaks also varied (Fig. 4). Indeed on occasions, particularly when small fluctuations of temperature or pH occurred, there was no obvious first peak but in the majority of these cases a second peak still developed (Fig. 4). In general, cells of the second peak were more resistant to small changes in environmental conditions than were the cells of the first peak.

Examination of smears made after various times in culture showed that the major changes during the first week were, firstly, an increase in the proportion of lymphocytes, from about 50% at the start of the culture to over 90% on day 6–7 (Fig. 5), and secondly, a rapid alteration in lymphocyte size. Virtually no small lymphocytes were present initially, but these rapidly appeared and by the 6th or 7th day practically all lymphocytes (>95%) were small (<7 μ diameter in smears) (Fig. 2b).

Table 3. The effect of different batches of foetal calf serum on cell proliferation on days 4 and 7 of organ culture. All batches were tested simultaneously in one experiment using DME with 15% FCS

Batch	Source	Cells per lobe ($\times 10^{-4}$)* Day 4	Day 7
969–1	CSL†	20·0	35·3
970–3	CSL	22·5	29·3
916–21	CSL	10·1	13·9
931–2	CSL	19·5	13·2
933–3	CSL	14·6	7·9
036	CSL	13·4	8·2
B 040	ALS†	8·5	9·7
B 041	ALS	18·7	36·7
B 042	ALS	28·4	52·5
50613	FLOW†	9·2	10·6

* The cultures were grown in organ culture dishes with two lobes per dish. Cell yields were obtained by haemocytometer counts on pooled cells obtained by trypsinizing 6–8 lobes per sample. The variation in cell number was due largely to variations in the proportion of lymphocytes seen.

† CSL, Commonwealth Serum Laboratories, Melbourne; ALS, Australian Laboratory Services; FLOW, Flow Laboratories.

Table 4. Comparison of the mean total cell counts per lobe ($\times 10^4 \pm 1$ SD) between 14 day foetal CBA thymus lobes cultured in either individual organ culture dishes (two lobes per dish in 1 ml medium) or in Gelfoam Millipore assemblies in 9 cm Petri dishes with 25–30 lobes per dish in 15–16 ml of medium

Days *in vitro*	Organ culture dishes	Gelfoam-Millipore assemblies
0	4 ± 1 (59)	
1	7 ± 3 (9*)	2 (1)
2	13 ± 2 (2)	ND
3	16 ± 4 (32)	15 ± 4 (4)
4	26 ± 22 (52)	31 ± 15 (6)
5	32 ± 11 (53)	48 ± 24 (13)
6	44 ± 18 (33)	62 ± 18 (16)
7	44 ± 22 (39)	68 ± 25 (9)
8	41 ± 30 (19)	73 ± 18 (5)
9	ND	ND
10	48 ± 27 (22)	66 ± 19 (4)
11	40 ± 25 (39)	70 ± 12 (9)
12	56 ± 30 (25)	84 ± 23 (22)
13	48 ± 17 (18)	93 ± 36 (10)
14	ND	65 ± 33 (3)

* Figure in brackets indicates the number of individual experiments per estimate. Each individual cell count was performed on the pooled trypinized cells of 6–8 lobes.

Analysis of the cell size distribution with the Coulter Counter confirmed these findings and showed that a gradual change in the size distribution occurred so that by day 6 the profile was similar to that of adult thymocytes, except that a shoulder of larger cells was present in the cultured cell population (Fig. 6). This shoulder is probably due to the non-lymphoid cells which represent a greater proportion of the total cells in the cultures than in the normally developing thymus. Coulter Counter profiles taken during the second week of culture showed that the cells of the second peak were marginally larger than those of the 1st peak, a difference which was also apparent in cell smears (Fig. 2c).

Electron-microscopy of suspensions of the cultured cells also showed that typical large and medium lymphocytes predominated during the first 48 h and were then progressively replaced by medium lymphocytes which in turn were replaced by small lymphocytes. It should be stressed that either plasmablasts or plasma cells were never seen. The non-lymphoid cells included fibroblasts, large phagocytic cells containing ingested cell debris, and epithelial cells which could be identified by their content of cytoplasmic bundles of microfibrils.

Immunofluorescence studies using *direct* staining with F1 R anti-MIg showed that surface labelled Ig-positive cells with the appearance of lymphocytes were rare (< 0·01%). However large cells showing cytoplasmic fluorescence were quite common particularly in older cultures. Radioautographs using ^{125}I-R anti-MIg showed that these large labelled cells were macrophages. Labelled lymphocytes were not seen (Fig. 2d).

Indirect immunofluorescence using anti-Thy-1 antiserum followed by F1 R anti-MIg showed that the majority (> 95%) of lymphoid cells by days 5–7 were positive and showed typical surface fluorescence which capped if cells were kept at room temperature. However, at all stages of the cultures, under standard conditions of labelling using 1 : 16 anti-Thy-1 followed by 1 : 16 anti-Ig, a range of intensity of fluorescence was seen ranging from barely discernable to very intense and identical with that seen with normal adult thymocytes. In general, in

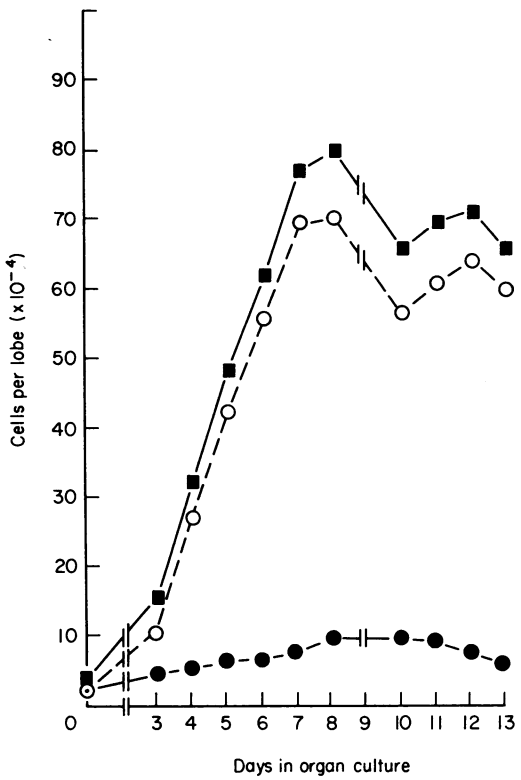

Figure 5. This figure shows the daily cell counts per lobe (■) in an experiment in which 14 day foetal thymus lobes were cultured on Millipore filters resting on Gelfoam in 9 cm petri dishes. The shape of the curve resembles those shown in Fig. 4, in that two peaks of cells are present on days 7–9 and 11–12. In addition the figure shows that the fluctuation is due primarily to variations in the numbers of lymphoid cells (○). The non-lymphoid cells, (●), consisting of macrophages, fibroblasts and epithelial cells show only a slight increase in number.

cultures which showed good early proliferation and a large first peak, the majority of lymphocytes were brightly stained whereas in cultures with a small first peak, and in all culture samples during the second week, the majority of lymphocytes were less intensely stained. However, it should be stressed that a wide spectrum of intensity of fluorescence of the lymphocytes was seen at all times after about the 3rd or 4th day of cultures and it was the relative numbers of brightly and faintly fluorescent cells which varied in the two peaks. Studies are currently in progress using the Fluorescence Activated Cell Sorter to accurately quantify these observations.

Small round cells which did not fluoresce under standard conditions with anti-Thy-1 were also present and accounted for 5–10% of the 'lymphoid cells' in older cultures. When indirect immunofluorescence using anti-TL serum was used, over 95% of the lymphocytes on days 5–7 were stained. However after this time the percentage of TL^+ cells gradually decreased (Fig. 7) so that by day 13 over 60% of the lymphoid cells were TL^-. It should be noted however that in these A/J cultures the majority of the lymphocytes were $Thy\text{-}1^+$ and some TL^+ cells were present even at day 13.

DISCUSSION

Previous reports from this laboratory described the morphology of the normal and cultured foetal mouse thymus (Mandel, 1970; Mandel & Russell, 1971). We have modified the culture method to obtain more prolific cell growth and have developed techniques to handle such minute organs in order to quantify and further characterize the changes which occur in the T-cell precursors as they proliferate and differentiate *in vitro*. The major aims of this study were to compare *in vitro* with *in vivo* development in order to determine whether the organ cultures were a reasonable model of normal development.

In the foetal thymus *in vivo* an exponential increase in total cell numbers occurs between 13 and 19 days gestation. During days 13 and 14 this increase is due about equally to lymphoid precursors and thymic epithelial and mesenchymal cells. Although a doubling in cell numbers occurs, no alteration in either cell morphology or in size distribution occurs in this period. Small lymphocytes are rare and most lymphocytes are medium or large. These observations suggest that in the early thymus, lymphocyte accumulation is due to inflow and/or to local proliferation of precursor cells. Previous studies have noted the apparently small contribution which intrathymic proliferation of the lymphocyte precursors makes to the lymphocyte pool during this period (Mandel & Russell, 1971). Presumably a 'maturation' of the thymic microenvironment is required before lymphopoiesis becomes prominent.

After this period a sharp alteration occurs in the cell population. The number and the proportion of lymphocytes increases rapidly and typical small cells predominate. Notable features of the late foetal period are the absence of SIg-positive cells and the

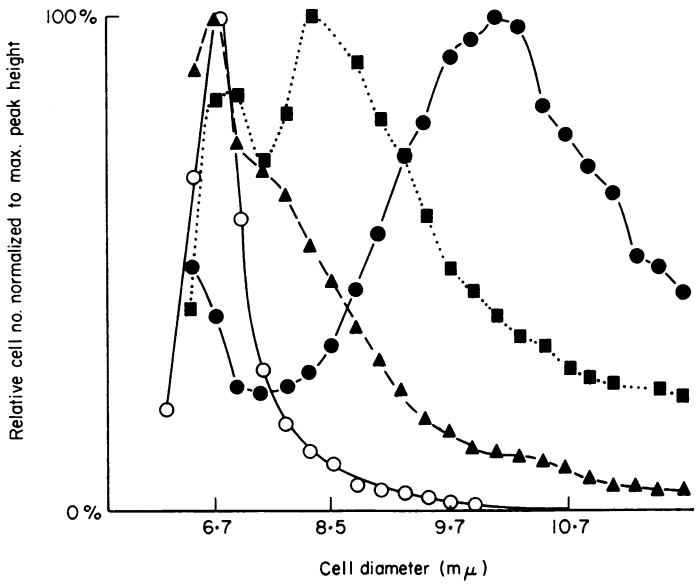

Figure 6. This figure shows a series of Coulter Counter size distribution profiles and illustrates the progressive decrease in cell size with increasing time in culture. The starting population of 14 day CBA foetal thymocytes (●) and the profile of normal adult thymocytes (○) are the same as those in Fig. 3. Cell size profiles from cultures taken on day 3 (■) and on day 6 (△) are illustrated. Day 3 *in vitro* is chronologically equivalent to day 16–17 of gestation while day 6 is equivalent to about day 20–21, i.e. to newborn cells.

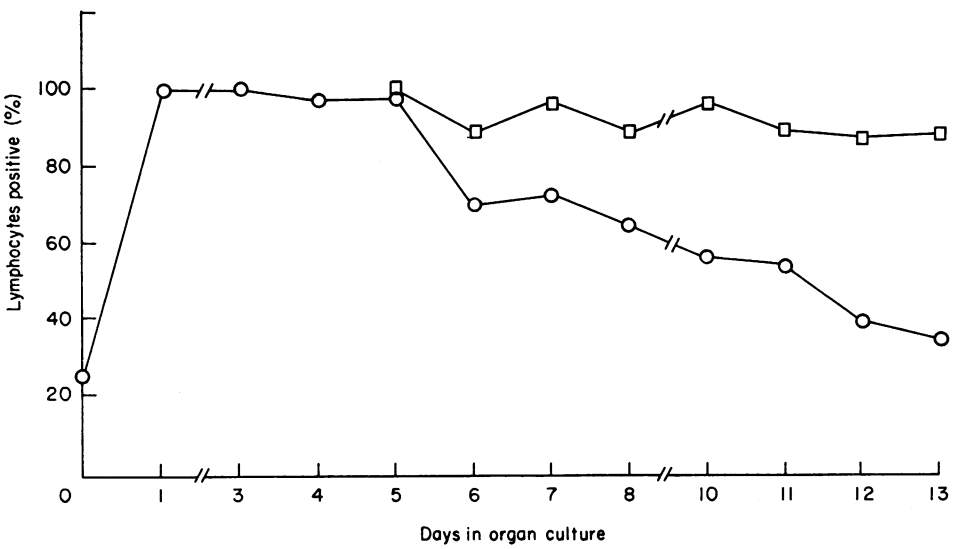

Figure 7. This diagram shows the decrease in the percentage of TL negative lymphocytes as a proportion of the total lymphocyte population over a 13 day culture period of A/J foetal thymus lobes. Note that the percentage of Thy-1 positive cells remains constant but that there is a progressive decrease in TL positive cells from day 5 onwards.

rapid increase in the proportion and intensity of staining of Thy-1 and TL positive cells. The absence of detectable Thy-1 positive cells in 14 day foetal liver and the presence of this antigen on most lymphoid precursors in the thymus at the same stage points to the rapid expression of this determinant once the T-precursors enter the thymic microenvironment. The expression of Thy-1 is weak initially and it cannot be demonstrated until day 16 by cytotoxicity (Owen & Raff, 1970; Phillips & Mandel, unpublished observations), although it can be seen by indirect immunofluorescence even on day 13 (Loor & Kindred, 1973). These data suggest that in the foetus expression of this antigen requires exposure to thymic influence and that this normally occurs within the thymus.

Under optimal culture conditions the foetal thymus generally parallels its development *in vivo*. Thus similar alterations occur in cell types, in their size distribution and in the expression of Thy-1 and TL antigens. A notable difference however, is the absence of a further inflow of T-cell precursors into the explanted thymus. In the normal foetal thymus a continuing influx of stem cells occurs and therefore, at any one time, cells at various stages of differentiation are present ranging from newly arrived progenitors to cells ready to die *in situ* or to leave the thymus for peripheral lymphoid tissues. The analysis of a normal thymus cell population is therefore complicated by the presence of cells at various stages of differentiation as well as by the difficulty of evaluating accurately the export of cells and the cell losses due to 'natural' and stress induced cell death. The use of a closed *in vitro* system in which corticosteroid mediated stress effects are absent, should make possible a precise analysis of T-cell differentiation. It should be stressed however that, in the absence of a vasculature, nutritional difficulties arise (as manifested by central necrosis) and these may limit the total proliferation of the lymphoid precursors. Nonetheless over 10^6 cells can be produced by day 6–7 compared with approximately 3×10^6 cells present in each lobe of a new born CBA mouse thymus.

In the organ cultures the sequential disappearance, initially of the large and then of the medium lymphocytes, and their replacement by large numbers of Thy-1^+ and TL^+ small lymphocytes shows that lymphocyte differentiation is indeed occurring. There is a large net increase in total cell numbers due mainly to the production of small lymphocytes. The non-lymphoid cells, although also increasing gradually, come to represent a relatively small proportion of the total cell pool. For example, from about 2×10^4 T-cell precursors present in a 14 day CBA foetal thymus lobe, $7–9 \times 10^5$ small lymphocytes may be produced by days 6–8 compared to 10^5 non-lymphoid cells produced from a similar initial number of their precursors. The large increase in lymphocytes in organ cultures contrasts with their usual decrease when dispersed cells are cultured, even in the presence of thymic epithelial cells (Mosier & Pierce, 1972). This difference may be due partly to the larger proportion of progenitor cells present in foetal tissue and/or to the presence of an intact inductive microenvironment. The latter is the more likely explanation since dispersed 14 day foetal thymus cells also show a net loss of lymphocytes over a period of a few days even though epithelial cells are still present (Mandel & Kennedy —unpublished observations).

A feature of these studies is the variation which can occur in lymphocyte proliferation, particularly during this first week. The batch of FCS used has a marked effect on the total cell number produced as do slight variation in pH and temperature. These effects are less evident during the second week of culture. Furthermore, the second wave of Thy-1^+ small lymphocytes can develop even when the initial population of Thy-1^+ TL^+ cells fails to appear. Studies in progress on the effects of hydrocortisone and thymidine suggest that the first population of small lymphocytes can be either prevented from developing or can be killed while still allowing the second population to appear (Mandel—manuscript in preparation). These data suggest that the two lymphocyte populations arise independently and support the concepts of Shortman & Jackson (1974); Schlesinger (1972) and Hopper & Shortman (1976). However the presence of TL^+ cells even in 13 day cultures suggests that some of the early appearing cells persist throughout the entire culture period. Whether the two populations are derived from a common precursor is however still unresolved.

Other workers, using basically similar organ culture methods, have not observed two peaks of lymphoid proliferation. Both Robinson & Owen (1976) and Juhlin & Alm (1976) show unimodal peaks of lymphoid proliferation, the former on day 6 with values of around 35×10^4 cells per lobe, whereas Juhlin & Alm (1976) give values of around 40×10^4 on day 15. The reason for the discrepancy

between our results and those of others is not known. It should be stressed however, that the cell proliferation produced in our cultures under optimal conditions is greater than that reported by others, and that maximum lymphoid proliferation, particularly during the first week of organ culture, is highly susceptible to environmental conditions. The lower cell numbers reported by Robinson & Owen (1976) and by Juhlin & Alm (1976) may be due to suboptimal culture conditions. The results of Juhlin & Alm (1976) on the appearance of Thy-1 and TL antigen also suggest this since they showed that far fewer cells expressed TL than Thy-1 on days 4–8.

The inability to demonstrate the presence of B cells in the cultured foetal thymus should make this system useful in studies of pure T-cell populations. SIg^+ cells are not demonstrable in 14 day foetal tissue either in the thymus or elsewhere (Nossal & Pike 1972; Spear, Wang, Rutishauser & Edelman, 1973) but both their precursors and other non-lymphoid haemopoietic precursors may be present in the thymus at this stage of development (Barg, Mandel & Johnson, 1978). A specific inductive microenvironment is probably obligatory for the full development of the precursor cells into their differentiated progeny (Metcalf & Moore, 1971). Our inability to demonstrate SIg^+ cells by a number of methods (immunofluorescence autoradiography and electron microscopy) as well as by the inability of these cultured cells to respond to the B-cell mitogen lipopoly-saccharide (Mandel, unpublished obs.) suggests that detectable numbers of mature B cells are not produced in these organ cultures. It can be argued that the use of trypsin to prepare single cell suspensions may remove Ig from the surface but controls using identical enzyme conditions on adult spleen cells showed that little if any SIg was removed. Furthermore, when foetal spleen fragments are cultured and dispersed with trypsin-EDTA, numerous SIg^+ cells can be demonstrated in a population which initially contained very few (DeLuca & Mandel, manuscript in preparation). The foetal thymus cells have however been shown to contain an Ig like material by surface iodination with lactoperoxidase followed by coprecipitation (Haustein, Marchalonis, Harris & Mandel, 1976).

This report therefore illustrates the use of an organ culture system for studying the development of differentiating lymphocyte populations from precursor cells. Under optimal conditions, organ cultures can be used to study the development of an essential arm of the immune system in a manner which resembles normal development. Studies which explore in more detail some of the points raised in this report are currently in progress (Luckenbach, Kennedy, Kelly & Mandel, 1978). In particular, we are seeking further evidence for the hypothesis that two separate Thy-1 positive lymphocyte populations are produced. We are also studying other antigenic and functional properties of cultured foetal lymphocytes in order to determine whether there is a dissociation during differentiation of functionally separate populations.

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