animals for maximal delay in appearance of hemagglutinin and coincident maximal cytotoxicity is 4 to 8 hours before injection of antigen.

1. Wust, C. J., Gall, C. L., Novelli, G. D., Science, 1964, v143, 1041.
2. Hanna, M. G., Wust, C. J., Lab. Invest., in press.
3. Brown, I. N., Nature, 1964, v204, 487.
4. Bukantz, S. C., Rein, C. R., Kent, J. F., J. Lab. Clin. Med., 1946, v31, 394.
5. Taliaferro, W. H., Taliaferro, L. G., J. Infect. Dis., 1957, v101, 252.
6. Brachet, J., Denis, H., DeVitny, F., Develop. Biol., 1964, v9, 398.
7. Lauter, H., Nakase, Y., Vanderberg, J., ibid., 1964, v9, 367.
8. Yaffee, D., Feldman, M., ibid., 1964, v9, 347.
9. Gross, P. R., Cousineau, G. H., Exp. Cell Res., 1964, v33, 368.
10. Scott, R. B., Bell, E., Science, 1964, v145, 711.
11. Braun, R., Mittermayer, C., Rusch, H. P., J. Cell Biol., 1963, v11, 9A.
12. Smiley, J. D., Heard, J. G., Ziff, M., J. Exp. Med., 1964, v119, 881.
13. Nathans, H. C., Bieber, S., Elion, G. B., Hitchings, G. H., Proc. Soc. Exp. Biol. and Med., 1961, v107, 796.
14. Cirkovic, D. M., Simic, M. M., Proc. Vth Yugoslav Conference on Radiobiology, Ljubljana, 1964.

Received December 16, 1964. P.S.E.B.M., 1965, v118.

Alkaline Phosphatase Activity of L-M Mouse Cells and Variants.* (30038)

SHEILA A. KEEFE, DONALD J. MERCHANT AND WILLIAM H. KELSEY
Department of Microbiology, University of Michigan, Ann Arbor

The usefulness of established lines of animal cells for studies in virology, radiation biology, oncology and many other fields is well known. The significance of results obtained with such systems depends, to a large extent, upon the ability to insure the constancy of characteristics of a given line. In a wild population, marked differences may exist among the cells in terms of biochemical characteristics, karyotype, virus sensitivity, etc., as demonstrated by the isolation of clonal strains with widely varying properties. Considerable variability can exist in the "same" strain of cells carried in different laboratories(1) or even as different stocks in the same laboratory(2), depending upon such factors as the choice of medium and conditions of cultivation.

The choice of markers for analysis of population changes depends on a number of factors including stability and ease of determination. Enzymes are particularly suitable for this purpose since they can be studied readily at the cellular level using a variety of histochemical or biochemical methods. One enzyme which appears to be promising is alkaline phosphatase (AP). Nitowsky and Herz(3) in a study of AP in cell cultures of human origin found the enzyme activity in Chang liver cells to be 2-200-fold greater than that in other cell lines studied. After several successive single cell platings of the Chang liver line, clonal strains were derived which showed significant differences in AP activity, and one such strain, morphologically distinguishable by its cell size had an activity only 1/40th that of the parent line.

Fortelius *et al*(4) described a clonal strain of giant HeLa cells which contained little or no AP activity in contrast to the parental line in which the enzyme activity varied from cell to cell but frequently was quite strong.

Studies by Cox and MacLeod(5) indicated that wide variability existed in AP activities among a number of mammalian cell lines and primary cultures. Of 6 uncloned HeLa lines from different laboratories 4 had demonstrable AP activity, and 2 had trace or no activity.

A technique was described by Maio and De Carli(6) in which clonal variants having

* This investigation was supported by USPHS Research Grant CA-03720-07 from Nat. Cancer Inst. and by Parke, Davis & Co., Detroit, Mich.

essentially no AP activity could be detected and isolated. One particular clone isolated from strain EUE (human embryonic epithelium) had less than 1/250th of the enzymatic activity of the parent strain and upon subsequent cultivation no positive colonies arose.

The present study reports the effect of 2 types of population selection pressure on distribution of AP activity in a line of L-M strain mouse fibroblasts and derived variants. Possible relationship with other cell markers is discussed.

Materials and methods. Cell lines. The L-M strain(7) is a subline of strain L (clone 929) mouse cells. It has been carried in modified medium 199(8) supplemented with 0.5% Bacto-peptone† and is designated in this report as L-M (199P). A nutritional variant has been cultured in double strength Eagle basal medium without supplements(9) and is called L-M (2XE). Each of these lines was tested for its *in vivo* growth capacity in weanling inbred C_3H mice by injecting approximately 1×10^7 log phase cells intramuscularly into the right foreleg. The mice usually were conditioned with cortisone acetate. The complete details of the animal experimentation are to be reported elsewhere.

The *in vivo* growth of the cells was evident by the appearance of a palpable mass in the area injected, designated as a nodule. Variant lines were obtained by excising the nodules, mincing them and trypsinizing the fragments to free the cells which were then grown as monolayers. Lines L-Ma through L-Mf were derived from nodules produced by L-M (199P) cells and were cultured in medium 199P. Lines L-Mg through L-Mi, derived from nodules resulting from injection of L-M (2XE) cells, were grown in 2XE medium. No antibiotics are employed for routine passage of cultures in our laboratory. All cell lines are checked at frequent intervals for the presence of *Mycoplasma* sp.

Assay procedures. Histochemical. Coverslip cultures in Leighton tubes were prepared by inoculating $1.5\text{-}2.0 \times 10^5$ cells in 1 ml of the appropriate medium. After 3-5 days incubation at 37°C, the coverslips were removed, fixed, and stained using a modification of the azo-dye method of Burstone(10). The substrate employed was naphthol AS-E phosphate‡ and Fast Blue RR salt§ was the coupler. A highly insoluble blue reaction product is formed when the naphthol released by hydrolysis of the substrate is coupled with the diazonium salt at the site of enzyme activity.

Quantitative. Monolayer cultures were harvested, by scraping with a rubber policeman, on the 6th or 7th day following inoculation of $1.5\text{-}2.0 \times 10^5$ cells/ml. The cells were counted, and the medium was removed following centrifugation. Approximately 1×10^7 cells/ml were extracted for 20 minutes in 0.25 M sucrose (15°C) containing 10% p(1,1,3,3-tetramethylbutyl) phenylpolyoxyethylene ethanol (Triton X-100).‖ The cell residue was removed by centrifugation and the supernatant fluid was frozen at −65°C until tested. AP activity of the Triton extract was determined using the substrate disodium p-nitrophenyl phosphate¶ in 1 M 2-amino-2-methyl-1-propanol-HCl** (AMP) buffer(11) at pH 10.6. The amount of p-nitrophenol (p-NP) released by hydrolysis of the substrate was determined at 410 mμ using a Beckman DU spectrophotometer. A standard curve was prepared for each experiment using known dilutions of p-NP(12,13). Appropriate dilutions of the samples were made when necessary to obtain optical density readings which would fall on the standard curve; each sample was run in duplicate. The phosphatase activity is directly proportional to the amount of p-NP liberated per unit time; one unit of AP is the amount of enzyme which liberates 1 μM of p-NP per ml per hour. Protein content of the extract was determined by the Lowry(14) method and expressed as mg/ml of undiluted sample. The specific activity of AP was taken as the ratio of the units of enzyme activity/ml to mg of protein/ml.

† Difco Laboratories, Detroit, Mich.

‡ Nutritional Biochemicals Corp., Cleveland, Ohio.

§ Allied Chemical, National Aniline Division, New York.

‖ Rohm and Haas, Philadelphia, Pa.

¶ Sigma Chemical Co., St. Louis, Mo.

** Distillation Products Industries, Div. Eastman Kodak Co., Rochester, N. Y.

Results. Fig. 1 through 6 show representative results of histochemical staining for AP. Passage 170 of L-M (199P) (Fig. 1) is non-uniform in its enzyme activity, with a number of cells non-reactive or only slightly active. On the other hand, the nutritional variant, L-M (2XE) in passage 108 (Fig. 4) is completely negative in AP activity. Isolates L-Ma and L-Mf from nodules produced by *in vivo* growth of L-M (199P) cells show more uniformity of activity within the population than the parent cells (Fig. 2 and 3).

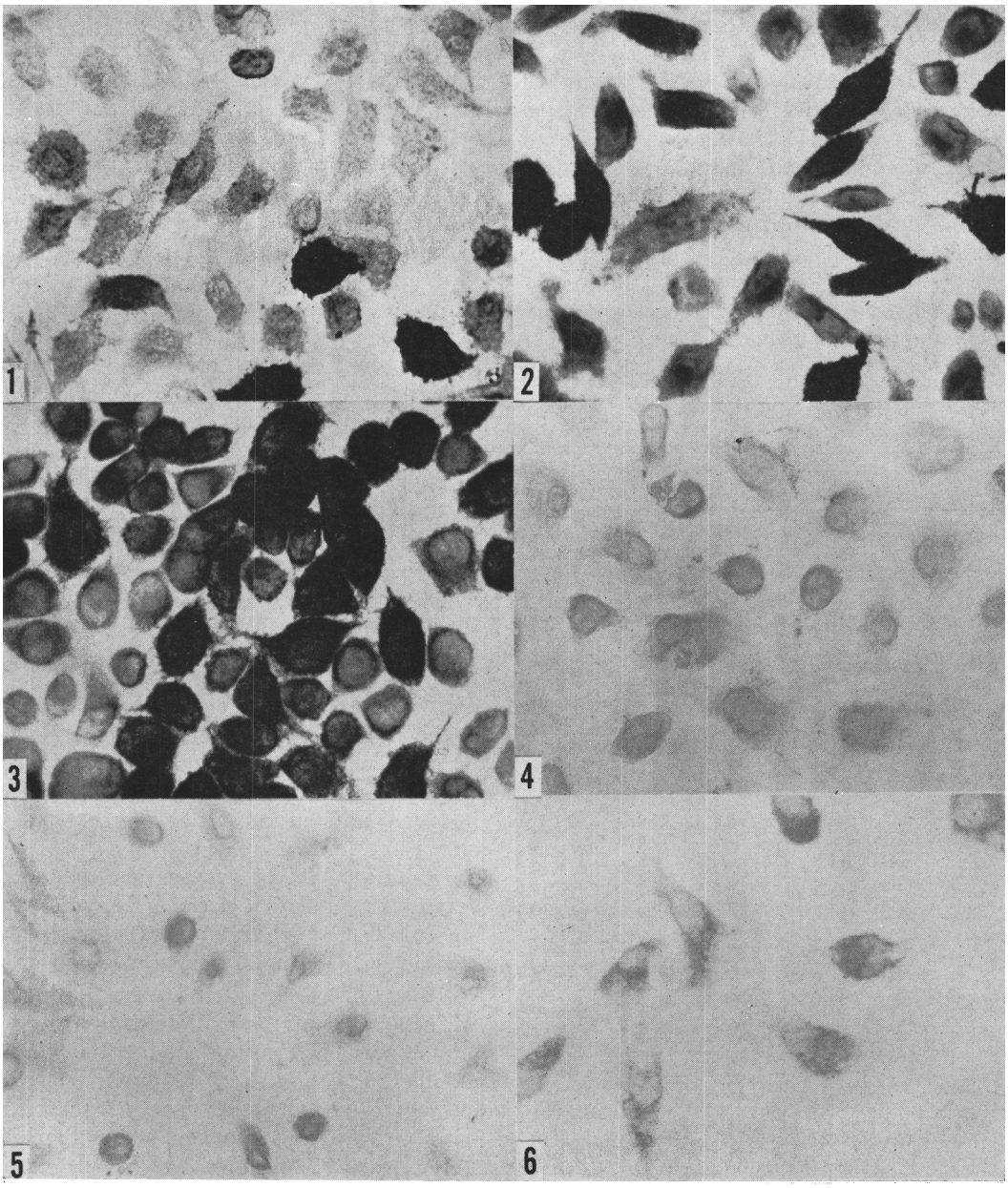

FIG. 1-6. Distribution of alkaline phosphatase activity in monolayer cultures of mouse cells. ×250.

Fig. 1. Passage 170 of L-M (199P) cells. Fig. 2. Passage 97 of L-Ma (199P) cells. Fig. 3. Passage 9 of L-Mf (199P) cells. Fig. 4. Passage 108 of L-M (2XE) cells. Fig. 5. Passage 4 of L-Mh (2XE) cells. Fig. 6. Passage 16 of L-Mi (2XE) cells.

The number of cells stained and the intensity of the stain is decidedly increased. It is possible to find in some passages of these isolates completely negative cells, but they rarely represent more than 5-10% of the population. The enzyme is stable, since the L-Ma line has been quite uniformly AP positive through at least 100 passages from the mouse. In contrast, cells from L-M (2XE) nodules are essentially negative when reisolated in culture; a few positive cells are seen occasionally. Fig. 5 and 6 show early passages of the L-Mh and L-Mi lines.

At various times during the *in vitro* growth of the lines described cells were frozen and stored in liquid nitrogen. For quantitative studies, several passages of a particular line were removed from the freezer at the same time and grown for one passage before being analyzed. Table I is a composite of the specific activities of the L-M (199P) line and its variants. The specific activities are averages of the activities for 2-5 consecutive passages in each culture interval, except where the time in culture was too short to permit more than 1 or 2 passages. The results show that L-M (199P) cells range from low to moderate in AP activity in a culture period covering 85 passages. On the other hand, with the exception of the interval represented by passages 43-52, the activity of L-Ma cells has been quite high. We have no adequate explanation for the low values found in passages 43-52. The L-Mc and L-Mf isolates, while studied less extensively, likewise demonstrate very high activity. The L-M (2XE) variant, by contrast, has essentially no AP activity, and remains negative during the *in vivo* passage, as seen from the analysis of 3 nodule isolates, L-Mg, L-Mh and L-Mi. A subjective evaluation of the intensity of histochemical staining gave results which were in general agreement with the quantitative data just presented.

TABLE I. Specific Activity of Alkaline Phosphatase in L-M Cells and Variants.

Cell line	Origin	Medium	Passage interval	Specific activity*
L-M	Reference 7	199P	84-88	1.43
			118-120	1.92
			127-129	1.76
			139-142	2.92
			152-154	5.03
			165-169	6.76
L-Ma	*In vivo* passage of 92LM (199P)	199P	7-10	22.10
			43-44	6.19
			49-52	6.52
			62-65	24.75
			76-79	17.37
			86-89	12.20
			91-92	15.14
L-Mc	*In vivo* passage of 98-101LM (199P)	199P	16-18	31.80
L-Mf	*In vivo* passage of 159LM (199P)	199P	3-5	25.05
			15-17	26.23
L-M	Reference 9	(2XE)	105-107	.01
			116-118	.01
L-Mg	*In vivo* passage of 128LM (2XE)	(2XE)	5	.0
L-Mh	*Idem*	(2XE)	Primary	.0
			6-7	.0
L-Mi	*In vivo* passage of 129-130LM (2XE)	(2XE)	14	.0
			16	.0

* Mean of specific activities for passages indicated. Specific activity = μM p-nitrophenol released per mg protein per hour at 37°C in AMP buffer, pH 10.6.

In studies of *in vivo* growth capacity of L-M (199P) and L-M (2XE) cells, distinct differences were noted between the two lines; in the case of L-M (199P), there is a selection of the population resulting in nodules comprised of cells more uniform in AP activity, with nodules appearing within 7-10 days in a large percentage of the animals; in the case of L-M (2XE), no such selection for alkaline phosphatase occurred, and nodules appeared no earlier than 3 weeks after injection and in a smaller percentage of animals.

Discussion. In the systems just described, 2 types of population selection pressure were studied: *viz.*, simplification of the nutritional environment and ability to survive and grow *in vivo*.

The history of the L-M lines in this laboratory has been one of gradual adaptation of the population from a highly complex medium (one containing embryo extract and horse serum) to a medium lacking protein (199 plus 0.5% peptone). This adaptation occurred over a period of 4 years and appeared to be accompanied by the emergence of marker chromosomes(7). Two years after the cells were established in 199P, some were

transferred to basal medium Eagle. On this occasion, and on many other subsequent transfers from the 199P medium, no significant lag was noted. The only apparent change was an increase in the population doubling time, which is characteristic of the medium in which the cells are being grown. Upon return of these cells to medium 199P, even after extended periods of growth in Eagle medium, an immediate resumption of the doubling time characteristic of this medium is noted.

The study of AP activity in L-M (2XE) cells was confined to a relatively short culture interval of passages 100-125, during which time the cells by both histochemical and biochemical methods were negative for alkaline phosphatase. Unfortunately, earlier passages were not available for assay. However, when L-M cells growing routinely in medium 199P are transferred to 2XE medium, there is a shift from a population which has moderate AP activity to one having decreased AP activity (unpublished observations). While these experiments have not been carried beyond 8 passages, and there are still a few AP positive cells present, one implication is that cells lacking in AP have a selective advantage in chemically defined medium and outgrow the other, positive cells. Nitowsky and Herz(15) observed that transfer of Chang liver cells into a different nutritional environment resulted in variation of AP activity suggesting the presence of diverse cell types in the population, one or more of which might enjoy a selective advantage and outgrow the other cells.

The technique of Maio and De Carli(6) in which clones can be first stained for AP and then isolated for subsequent culture, suggests a means of testing this hypothesis. At present, such a study is hampered by inability to clone the L-M cell line in protein-free medium. While Cox and MacLeod(5) have shown that lack of AP poses no hardship for a cell line in terms of its ability to grow *in vitro,* Maio and De Carli(16) reported that lines with very low AP activity grew at a slower rate than positive lines under the same culture conditions.

In vivo passage of L-M (199P) cells, which *in vitro* show wide variation in AP activity from cell to cell, with a large number nonreactive or only slightly active, results in a selection of cells more homogeneous in AP activity with many which are strongly positive for the enzyme. L-M (2XE) cells, however, do not acquire AP during *in vivo* passage. It thus appears unlikely that the results can be explained by enzyme induction, since in both instances, the cells were subjected to the same influences, including hormones. Likewise those isolates which do show increased enzyme levels should, in the absence of an inducer, return to normal low levels within a few passages *in vitro.* That this is not the case is demonstrated by the high activity of L-Ma cells after almost 100 passages *in vitro.* On the other hand, the possibility that L-M (2XE) cells have lost an inherited ability to be induced, cannot be ruled out. Other investigators(5,6,16,17) have shown that AP can be induced by prednisolone or by substrates *in vitro,* and such studies are suggested for this system.

Various markers, including drug and radiation resistance and virus sensitivity, have been described for clonal derivatives of tissue culture cell lines(18). In some cases, the karyotype of the clone differed from that of the parent line, and the marker trait was stable in the absence of the selective force, thus leading to the hypothesis that such characteristics are genetically determined.

In *Escherichia coli,* AP is controlled by 3 genes—one specifies the structure of the enzyme while the other 2 regulate its rate of formation dependent upon the concentration of orthophosphate in the medium(19).

In mammalian systems much less is known concerning the regulation of AP. Correlations have been made regarding increase or decrease of leucocyte AP levels with changes in genetic complement(20-23). Two recent studies(24,25) have described distinctive karyotypes for clones of human cell cultures which differ markedly from the parent line in AP activity. Thus there is some evidence indicating that mammalian AP also is genetically determined.

The L-M (199P) cell line and 3 of its variants, L-Ma (199P), L-M (2XE) and L-Mi (2XE) were selected for detailed chromosome analysis. The significant features of

this work, which will be reported elsewhere, are 1) the downward shift in the frequency distribution of the E marker chromosome in both L-M (199P) and L-Ma (199P) cells, and 2) the emergence of 2 new marker chromosomes, called G and H, in both L-M (2XE) and L-Mi (2XE) cells(26). While these shifts parallel changes in AP activity and in ability to produce nodules in inbred C_3H mice, it would be premature to speculate on a cause and effect relationship at this time.

One other group of enzymes has been studied in some of the cell lines described here, *viz.*, non-specific esterases. While histochemically all the lines appeared to be equal in activity the zymogram technique showed distinct differences both in number and mobility of esterases among L-M (199P), L-Ma (199P) and L-M (2XE) cells. The latter had 2 slow moving bands which were not seen in either of the other lines. What relationship these bands may share with the absence of AP is not known. Attempts to use this technique for the study of AP were largely unsuccessful because the enzyme did not migrate well in the starch. Cellulose acetate or polyacrylimide gel may prove to be more satisfactory for separation of AP.

Summary. Two types of population selection pressure *viz.*, simplification of the nutritional environment and ability to survive and grow *in vivo*, were seen as effecting the distribution of alkaline phosphatase activity in L-M strain mouse fibroblasts and derived variants. L-M cells growing in medium 199 supplemented with peptone have moderate AP activity as determined quantitatively; histochemically, the population includes a number of cells with little or no activity as well as cells with moderate to high activity. A variant of these cells growing in a chemically defined medium has no measurable AP activity. Distinct differences were noted between these 2 lines when their *in vivo* growth capacity in inbred C_3H mice was studied: in the line having moderate AP activity, there is a selection of the population resulting in nodules comprised of cells of high AP activity, with these nodules appearing within 7-10 days in a large percentage of the animals; in the line having no initial enzyme activity, there was no such selection for AP, and nodules appeared no earlier than 3 weeks after injection and in a smaller percentage of animals.

1. Hsu, T. C., Biological Contributions, Univ. of Texas, Austin, 1959, (Fall), 129.

2. Sanford, K. K., Merwin, R. M., Hobbs, G. L., Fioramonti, M. C., Earle, W. R., J. Nat. Cancer Inst., 1958, v20, 121.

3. Nitowsky, H. M., Herz, F., Nature, 1961, v189, 756.

4. Fortelius, P., Saksela, E., Saxén, E., Exp. Cell Res., 1960, v21, 616.

5. Cox, R. P., MacLeod, C. M., J. Gen. Physiol., 1962, v45, 439.

6. Maio, J. J., De Carli, L. L., Nature, 1962, v196, 600.

7. Hsu, T. C., Merchant, D. J., J. Nat. Cancer Inst., 1961, v26, 1075.

8. Eidam, C. R., Merchant, D. J., Exp. Cell Res., accepted for publication.

9. Merchant, D. J., Hellman, K. B., Proc. Soc. Exp. Biol. and Med., 1962, v110, 194.

10. Merchant, D. J., Kahn, R. H., Murphy, W. H., Handbook of Cell and Organ Culture, 2nd ed., Burgess Publishing Co., Minneapolis, 1964, 194.

11. Lowry, O. H., Roberts, N. R., Wu, M., Hixon, W. S., Crawford, E. J., J. Biol. Chem., 1954, v207, 19.

12. Sigma Technical Bull., No. 104, 1961.

13. Linhardt, K., Walter, K., Methods of Enzymatic Analysis, H. U. Bergmeyer, Ed., Academic Press, New York, 1963, 783.

14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.

15. Nitowsky, H. M., Herz, F., Proc. Soc. Exp. Biol. and Med., 1961, v107, 532.

16. Maio, J. J., De Carli, L., Biochem. Biophys. Res. Comm., 1963, v11, 335.

17. Cox, R. P., Pontecorvo, G., Proc. Nat. Acad. Sci., 1961, v47, 839.

18. Merchant, D. J., Neel, J. V., Ed., Approaches to the Genetic Analysis of Mammalian Cells, Univ. of Michigan Press, Ann Arbor, 1962.

19. Garen, A., Echols, H., Proc. Nat. Acad. Sci., 1962, v48, 1398.

20. Alter, A. A., Lee, S. L., Pourfar, M., Dobkin, G., J. Clin. Invest., 1962, v41, 1341.

21. King, M. J., Gillis, E. M., Baikie, A. G., Lancet, 1962, v2, 1302.

22. Valentine, W. N., Beck, W. S., J. Lab. Clin. Med., 1951, v38, 39.

23. Nowell, P. C., Hungerford, D. A., Science, 1960, v132, 1497.

24. Konisberg, U. R., Nitowsky, H. M., J. Nat.

Cancer, Inst., 1962, v29, 699.

25. De Carli, L., Maio, J. J., Nuzzo, F., ibid., 1963, v31, 1501.

26. Merchant, D. J., Giles, R., Masselink, E., Keefe, S. A., Excerpta Med. (Sect. I), 1964, v18, 52.

Received December 22, 1964. P.S.E.B.M., 1965, v118.

Long Term Culture of Thymic Cells from Newborn Mouse Thymus Fragments.* (30039)

L. TOMATIS AND L. WANG (Introduced by P. Shubik)

Division of Oncology, Chicago Medical School, Chicago, Ill.

Short term cultures from normal thymus cells have been used by many investigators for various purposes(1,2,3,4,5,6). Mouse lymphomatous thymic lymphocytes were grown successfully *in vitro* for over 300 days by Ioachim and Furth(7); cultures of embryonal and normal adult thymic cells, however, resulted in a good growth of only reticulum cells, while the lymphocytes disappeared completely within 2-3 days. The persistence of thymic lymphomatous lymphocytes for as long as 32 weeks was observed by Hays(8) in diffusion chambers implanted i.p. in isogeneic and allogeneic mice, whereas chambers containing normal adult thymus fargments were devoid of lymphocytes after one week. In the experiments here reported minute newborn thymus fragments were cultured *in vitro* with the purpose of obtaining a long term culture of thymic lymphocytes. Two different techniques of explanation of the thymus fragments were used.

Materials and methods. Swiss non-inbred, and inbred MA and C57 mice were used as source of the thymuses. Eighteen- to 40-hour-old mice were killed with ether, and as soon as breathing stopped the thymus was removed aseptically to a Petri dish containing Hanks' BSS. After washing, the thymuses were individually transferred to second Petri dishes containing Hanks' BSS and finely minced. The fragments were then transferred to Leighton tubes (Bellco Glass Co., Vineland, N. J.) into which coverslips had been previously inserted.

The culture medium was Hanks' BSS with phenol red as indicator, plus vitamins, glutamin, essential amino acids, and was supplemented with 11% fetal bovine serum (Microbiological Associates, Inc., Bethesda, Md.). No antibiotics were used. Two methods of culturing the fragments were employed.

In a first series of experiments one or two drops of chicken plasma (Difco) were pipetted on the cover slip before inserting the thymus fragments into the tubes. The plasma solidified rapidly after transfer of the fragments and the medium was then added. This procedure favored a rapid and firm adhesion of the fragments to the glass. The medium was first changed after 4-5 days, and at this time it was completely discarded. It seemed advantageous for the further growth of the cultures that as much as possible of the debris from dead or degenerating lymphocytes should be removed. After this first change the medium was changed regularly every week, but the old medium was no longer completely discarded; approximately ¼ of it was left in the tubes and fresh medium added.

In the second series of experiments the thymus fragments were inserted into the tubes without previously adding chicken plasma. The thymus fragments floated free in the medium and were never attached to the glass. The medium was changed the first time as described above, but 4-5 days later the fragments were transferred to a new tube. From then on the medium was changed regularly every week, always leaving a part of the old medium in the tubes.

The tubes were maintained at 37°C in an incubator without CO_2 and were observed daily using an inverted microscope. At various times the cover slips were removed from

* This work was supported by grant CA-04665 from Nat. Cancer Inst., Nat. Inst. Health, U.S.P.H.S.