identical gradient. The cells were mixed, freed from the Ficoll (by centrifugation after dilution), resuspended in fresh culture medium, and layered upon another identical gradient. When centrifuged once again, the cells were distributed into two distinct bands (Fig. 1C) corresponding to their positions on the original gradient. The return of the cells to their original position indicates that the Ficoll gradient does not alter their density significantly. The increased wall effects seen in this gradient were the result of incomplete resuspension of the cells after their centrifugation to a pellet to remove the Ficoll of the first gradient. Complete dispersion of the cells subsequently was achieved with a Dounce homogenizer without apparent cell damage.

Microscopic examination of fractions, taken progressively down a gradient such as shown in Fig. 1A, revealed that cells gradually increased in size from small morphologically uniform daughter cells at the top to larger dividing cells at the bottom of the zone of buoyant cells. Thus, *Chlorella* cells appear to increase in density as they develop through their cell cycle.

A gradient was then designed to allow floatation of daughter cells and sedimentation of most intermediate-age and mother cells (Fig. 1D). Such gradients could be heavily loaded [up to approximately 2 g of cells (fresh weight) per 35 ml of gradient] to insure a good yield of uniform daughter cells. The daughter cells taken from such gradients exhibited a high degree of synchrony, a periodic (step) increase in total cellular DNA, and approximately an exponential increase in total cellular protein during a synchronous cell cycle (Fig. 2). When the pelleted (older) cells were taken along with the buoyant daughter cells, the cell number increased exponentially as in the untreated asynchronous culture, an indication that the Ficoll density-gradient procedure did not of itself induce synchrony.

This isopycnic density-gradient procedure potentially offers another rapid experimental approach to biochemical investigations of the cell cycle. If cells are buoyant in the gradient materials employed and if they increase continuously in density as they develop through their cell cycles, it should be possible to reveal how biochemical parameters change during a cell cycle merely by making cell counts and biochemical analyses down the zone of buoyant asynchronous cells.

To perform such studies, however, the cultural conditions must be closely controlled. *Chlorella* cells of all ages increased in density as their growth rate was increased by illumination with a higher light intensity. Under 22,000 lu/m^2 the cell cycle length remained about the same, but the division number of the cells increased to approximately eight. The density of dividing mother cells increased to the point that they could not be floated in the highest density of Ficoll tested (58 percent), but daughter cells and those of intermediate age still could be floated in gradients running from 25 to 40 percent Ficoll.

One advantage of this equilibrium centrifugation procedure is that the cells are maintained at 2° to 3°C from the time of harvest until they are again suspended in culture medium that had been equilibrated to the temperature necessary for initiating cell growth. At this low temperature the metabolism of the cells is not as likely to be perturbed as may be the case in intermittent-illumination procedures (*7*) used to synchronize *Chlorella*.

From the review by Morse (*8*) dealing with methods being tested and used for fractionation or separation of cells from tissues or suspension cultures, it appears that equilibrium centrifugation as a means of selection of synchronous daughter cells for cell cycle studies has not been reported.

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References and Notes

1. J. M. Mitchison and W. S. Vincent, in *Cell Synchrony—Studies in Biosynthetic Regulation*, I. L. Cameron, G. M. Padilla, Eds. (Academic Press, New York, 1966), pp. 328–331; P. Tauro and H. O. Halvorson, *J. Bacteriol.* **92**, 652 (1966).
2. R. Sinclair and D. H. L. Bishop, *Nature* **205**, 1272 (1965); T. O. Fox and A. B. Pardee, *Science* **167**, 80 (1970).
3. R. C. Leif and J. Vinograd, *Proc. Nat. Acad. Sci. U.S.* **51**, 520 (1964).
4. C. Sorokin, *Physiol. Plant.* **10**, 659 (1957).
5. Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.
6. T. K. Lakshmanan and S. Lieberman, *Arch. Biochem. Biophys.* **53**, 258 (1954).
7. R. R. Schmidt, in *Cell Synchrony—Studies in Biosynthetic Regulation*, I. L. Cameron, G. M. Padilla, Eds. (Academic Press, New York, 1966), pp. 189–235.
8. R. K. Morse, *Bull. Inst. Cell. Biol. (Univ. Conn.)* **11** (No. 4), 1 (1969).
9. T. A. Hare and R. R. Schmidt, *Appl. Microbiol.* **16**, 496 (1968).
10. K. Burton, *Biochem. J.* **62**, 315 (1956).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
12. Supported in part by grants from NSF (GB-17305), PHS (5 SO5 FR07095-02), and NASA (NGR 47-004-006).
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Quantitation of Strain BALB/c Mouse Peritoneal Cells

Abstract. *Total and differential counts of the peritoneal cells of male and female BALB/c mice aged 10 days to over 2 years demonstrate that the increase in cell number that occurs in mice over 2 months old is due entirely to an increase in lymphocytes. The number of peritoneal macrophages in BALB/c females is maintained at a constant level for 22 months. The stability of the macrophage population in contrast to the increase in numbers of lymphocytes suggests that the body pools of these two cell types are not related.*

Mouse peritoneal cells are now being used in studies concerned with the uptake and processing of exogenous materials (*1*), especially antigens (*2*). It has been recognized that this cell population consists mainly of macrophages and lymphocytes, and it is known that the proportion of cells is altered by the introduction of foreign materials into the peritoneal cavity (*3–5*); to our knowledge, no information is available concerning the variation in the number of each cell type that is related to age, sex, and strain (*6*). The results presented here indicate how age and sex influence the normal peritoneal cell population of the strain BALB/c mouse.

Data were derived from conventional and germ-free mice obtained from the breeding facilities of the National Institutes of Health. The total number of peritoneal cells was estimated by a modification of the method employed by Hauschka *et al.* (*7*) and Klein and Revesz (*8*), in which the cells were washed out of the peritoneal cavity by repeated rinsings with phosphate-buffered saline into graduated cylinders containing aqueous solutions of citric acid and crystal violet in amounts such that the final concentration in the washings was 0.1*M* and 0.04 percent, respectively. Between 5 and 25 ml of saline were used for rinsing, according to the age of the animal. The gradu-

ates were shaken vigorously at intervals over a period of not less than 20 minutes to disperse the cells, and the nuclei in at least two aliquots from each graduate were counted in a hemacytometer.

Preparations for differential counts were made by removing 1 to 5 μl of saline-diluted peritoneal fluid from the peritoneal cavity and placing droplets on saline-moistened HA Millipore filters, 5 mm in diameter. The filters were fixed 30 minutes in 10 percent formalin in 95 percent ethanol, stained in hematoxylin-eosin-azure, run up through alcohols and toluene, and permanently mounted on glass slides with plastic resin. At least 1000 cells from three or more separate samplings from each mouse were enumerated to compile the differential cell counts.

Preservation of cell morphology in the filter preparations permitted the recognition of the various cell types with a high degree of accuracy. Lymphocytes and macrophages constituted at least 95 percent of the peritoneal cells, while mast cells, polymorphonuclear leukocytes, and a few myeloblasts (more prominent in younger animals) made up the remainder of the cell types. If other cells were present, they could not be recognized. Contrary to other reports (*3*), we were not able to identify mesothelial cells in the peritoneal fluids of these normal animals. Medium lymphocytes predominated in animals under 4 weeks of age while small lymphocytes were rarely seen. In older animals, small, medium, and large lymphocytes were present. Polymorphonuclear leukocytes were always present but at levels of a few tenths of a percent. Mast cells in young animals were small and sparsely filled with granules; in older animals, they were large and heavily packed with granules. Some of the macrophages from mice of all ages contained phagocytosed material.

It was not possible to be confident of total and differential counts from mice younger than 10 days of age but, as an order of magnitude, counts from 1-day-old female mice ranged from 0.04 to 0.09 $\times 10^6$ total cells, while differential counts on mice of this age indicated that macrophages comprised about 85 percent; lymphocytes, 6 percent; and mast cells, polymorphonuclear leukocytes, and myeloblasts, 9 percent of the cells. The total number of peritoneal cells increased rapidly from this number at birth to about 3 $\times$ 10^6 cells at 4 weeks, a 50-fold increase (Table 1). After a further two- to threefold increase during the next 4 to 6 weeks, the total peritoneal cell population was characterized by a slow rise in cell number for the duration of the life of the animal. In some female mice between 1 and 2 years of age, a dramatic infiltration of lymphocytes occurred.

Table 1. Total peritoneal cells in BALB/c mice.

Age (days)	Total cells $\times 10^{-6}$ (range)	Animals (No.)
	Conventional females	
10	0.43 (0.34–0.49)	8
20	1.4 (1.1–1.6)	6
29	2.1 (1.3–2.8)	7
70	7.4 (6.2–9.4)	7
122	8.8 (6.7–10.3)	8
162	13.1 (9.8–16.4)	8
212	13.7 (9.8–19.0)	8
398	19.0 (11.9–39.1)	8
477	19.6 (11.2–31.8)	8
750	34.0 (12.5–56.1)	8
	Germ-free females	
10	0.41 (0.35–0.50)	6
21	1.2 (0.79–1.4)	8
62	4.7 (4.1–5.0)	4
217	14.5 (13.2–16.1)	5
240	17.9 (15.6–19.2)	3
284	18.6	1
	Conventional males	
10	0.36 (0.31–0.42)	5
66	2.3 (2.1–2.6)	3
235	7.7 (6.7–9.2)	8
467–473	7.5 (1.6–9.6)	13
	Germ-free males	
62	2.1 (1.8–2.6)	4
210	3.9 (3.8–4.1)	2

It can be seen from the differential counts (Figs. 1 and 2) that, after the first 3 months of age, the increase in peritoneal cells in female mice was due entirely to increases in the numbers of lymphocytes. By contrast, the population of macrophages in the peritoneal cavity was quite stable. Thus, after 10 days of age, the total macrophages increased approximately tenfold, while at the same time the lymphocytes increased several hundred to 1000-fold. In 27 conventional female mice between the ages of 70 and 750 days, the total macrophages averaged 2.3 million with a range of 1.5 million to 3.0 million.

The peritoneal fluid of male mice (Fig. 2) contained fewer cells than that of females, and this was due largely to a reduced number of lymphocytes. Old male mice did not show the infiltration of lymphocytes that characterized the peritoneal fluid of old females. Finally, it can be seen (Fig. 2) that rearing mice in a germ-free environment did not appear materially to affect the total number or proportions of peritoneal cells.

These results show that the peritoneal lymphocyte population of the BALB/c mouse is influenced by age and sex, while the number of macrophages is remarkably constant. The influence of the thymus on the postnatal maturation of the lymphatic system is well documented (*9*), and the 50-fold increase in peritoneal lymphocytes in the first 4 weeks of life correlates well with the 30-fold increase in total cell number observed in the thymus of C_3H_f mice during the first 2 weeks of life (*10*). The slow but definite rise in numbers of lymphocytes in the peritoneal fluid of mice between 3 and 22 months perhaps reflects a gradual increase in the size of the pool of long-lived lymphocytes as the animals grow older.

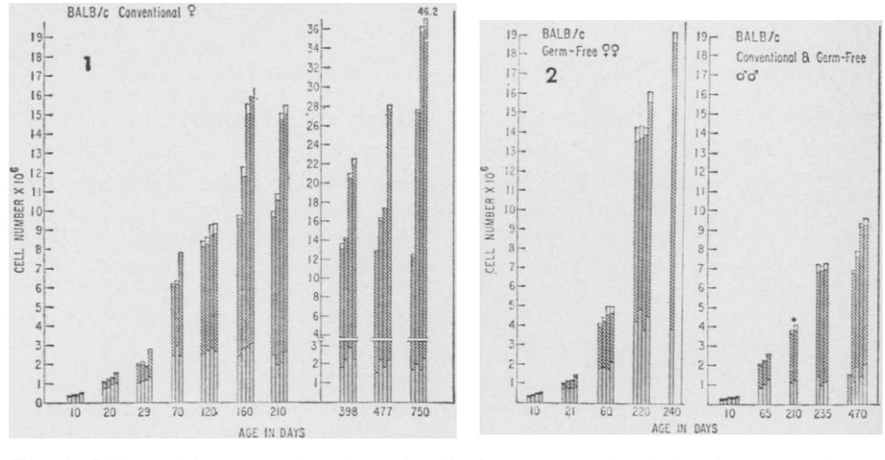

Fig. 1. Differential counts of peritoneal cells from conventional female mice. *Macrophages* are represented by the stippled bars at the bottom, *lymphocytes* by the hatched bars, *polymorphonuclear leukocytes* and *mast cells* by the open bars at the top. Each bar represents data from a single mouse. Fig. 2. Differential counts of peritoneal cells from germ-free female and conventional and germ-free male mice. Differential counts from two germ-free males are indicated by the asterisk. Cells are represented as described in legend for Fig. 1.

Gowans and Knight (*11*) have shown that, in the rat, lymphocytes recirculate from blood to thoracic duct lymph via the postcapillary venules of lymph nodes, spleen, and intestinal Peyer's patches. Since the peritoneal space encloses most of these viscera, the possibility that the lymphocytes of the peritoneal fluid of mice also participate in the recycling deserves to be tested.

Little is known about the origin and homeostatic control of the normal peritoneal macrophage population in mice. The bulk of the literature relates either to the mobilization of these cells in response to various stimuli or the repopulation of peritoneal fluid in irradiated animals (*4, 12, 13*). It is clear that peritoneal macrophages normally have a very low mitotic rate in vivo and in vitro (*14*), but a rapidly proliferating population can be elicited by injecting antigen intravenously (*15*), or foreign materials intraperitoneally (*13*). Volkman (*13*) provides evidence that these rapidly dividing macrophages are derived from the bone marrow but their relationship to the normal peritoneal macrophage population is not clear. Experiments by North (*16*) indicate that rapidly dividing macrophages are not necessarily mobilized from a distant site since a high percentage of mature resident peritoneal macrophages can be stimulated to divide by the intravenous injection of *Listeria monocytogenes*. What emerges from the present quantitative study is the fact that the peritoneal macrophage population is numerically stable in normal adult mice. The numerical stability of the peritoneal macrophage in comparison with the numerical fluctuations of the peritoneal lymphocyte suggests that there is no direct relationship between the body pools of these two types of cells, a conclusion similar to that reached by Volkman (*13*).

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References and Notes

1. B. A. Ehrenreich and A. A. Cohn, *J. Reticuloendothelial Soc.* **5**, 230 (1968).
2. M. Fishman, *J. Exp. Med.* **114**, 837 (1961); B. F. Argyris, *J. Immunol.* **99**, 744 (1967); ———, and B. A. Askonas, *Immunology* **14**, 379 (1968); W. Braun and L. J. Lasky, *Fed. Proc.* **26**, 642 (1967); A. E. Bussard and M. Lurie, *J. Exp. Med.* **125**, 873 (1967); E. R. Unanue and B. A. Askonas, *ibid.* **127**, 915 (1968).
3. G. Klein, *Exp. Cell Res.* **11**, 518 (1951); M. D. Felix and A. J. Dalton, *J. Nat. Cancer Inst.* **16**, 415 (1955).
4. J. Padawer and A. S. Gordon, *Anat. Rec.* **124**, 209 (1956); R. S. Speirs and M. E. Dreisback, *Blood* **11**, 44 (1956).
5. E. Shelton and M. E. Rice, *Amer. J. Anat.* **105**, 281 (1959).
6. Total and differential counts of peritoneal cells from strains C_{57}Bl and DBA/2 mice are available from E. Shelton, National Cancer Institute, upon request.
7. T. S. Hauschka, S. T. Grinnell, L. Revesz, G. Klein, *J. Nat. Cancer Inst.* **19**, 13 (1957).
8. G. Klein and L. Revesz, *ibid.* **14**, 229 (1953).
9. V. Defendi and D. Metcalf, Eds., *The Thymus* (Wistar Institute Press, Philadelphia, 1964), 139 pp.
10. A. A. Axelrad and H. C. van der Gaag, *J. Nat. Cancer Inst.* **28**, 1065 (1962).
11. J. L. Gowans and E. J. Knight, *Proc. Roy. Soc. London Ser. B* **159**, 257 (1964).
12. H. Balner, *Transplantation* **1**, 217 (1963); D. F. Cappell, *J. Pathol. and Bacteriol.* **33**, 429 (1930); J. W. Goodman, *Blood* **23**, 18 (1964); M. Virolainen, *J. Exp. Med.* **127**, 943 (1968).
13. A. Volkman, *ibid.* **124**, 241 (1966).
14. C. A. Mims, *Brit. J. Exp. Pathol.* **45**, 37 (1964); Y. T. Chang, *J. Nat. Cancer Inst.* **32**, 19 (1964).
15. I. J. Forbes, *J. Immunol.* **96**, 734 (1966); K. K. Khoo and G. B. Mackaness, *Australian J. Exp. Biol. Med. Sci.* **42**, 707 (1964).
16. R. J. North, *J. Exp. Med.* **130**, 299 (1969).

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Extinction in Goldfish: Facilitation by Intracranial Injection of RNA from Brains of Extinguished Donors

Abstract. *Extracts rich in RNA were prepared from the brains of goldfish that had acquired and then extinguished a light-signaled avoidance to shock in an aquatic shuttle-box. Recipient fish injected intracranially with such extracts extinguished the response significantly faster than those injected with extracts prepared from brains of naive donors.*

Investigators attempting to demonstrate effects of biochemical transfer from trained donors to naive recipients have used, almost exclusively, acquisition paradigms. A review of the literature on vertebrates revealed no transfers, nor attempted transfers, of experimental extinction. The injection of brain homogenates from donor rats given successive trials for acquisition, extinction, and reacquisition of a response facilitates indicant responses in naive recipient rats, as compared with controls (*1*). However, no attempt has been made to transfer extinction itself. The successful transfer of some extinction effect, besides extending the generality of the biochemical transfer phenomenon, would be invulnerable to any criticism that the effect was due to some general activating or energizing property of the extract employed. Furthermore, a successful transfer of extinction would yield information about the nature of the extinction process itself.

That my attempt might succeed was suggested by the positive results obtained by Ungar (*2*) in his chemical transfers of another decremental effect, the habituation of the startle response to auditory and air-puff stimuli. Habituation and experimental extinction appear to be quite similar processes, a major difference being that repeated presentation of stimuli leads to a decrease in an unconditional response in the former case, and in a conditional response in the latter. Goldfish were chosen as experimental subjects because they offer many advantages for biochemical research (*3*), and because Fjerdingstad (*4*) using a shuttle-box avoidance task had already demonstrated an acquisition transfer effect in the species.

The first experiment was a systematic replication of Fjerdingstad's acquisition study. The apparatus and procedure were identical to those used in the acquisition phase of the extinction experiment described below. Sixteen goldfish were given 8 days of training for acquisition of shock avoidance in an aquatic shuttle-box; a daily session consisted of 20 trials. The performances of these experimental donors, in mean percentage of avoidance responses, for the 8 days were, respectively: 18.75, 53.35, 75.31, 90.00, 93.75, 91.25, 93.44, and 92.50. These animals, along with 16 naive control donors, were killed 24 hours after the last trial; extracts of brain RNA were prepared according to the method described below. Such extracts might best be described as RNA-rich, since a Folin-Ciocalteu test reveals the presence of protein or polypeptide, in addition to RNA. Two groups of 14 naive recipient fish, chosen randomly, were injected intracranially with the equivalent of the extract of one brain from either a control or a trained fish in 40 μl of $10^{-3}M$ saline. All animals were tested in the shuttle-box without reinforcement 48, 72, 96, and 120 hours after injection. A daily test consisted of 20 trials with light only (without shock) in which avoidance responses were scored (Table 1). Performance of the fish injected with brain extract from trained fish was reliably greater than that of fish injected with extract from control fish at the first