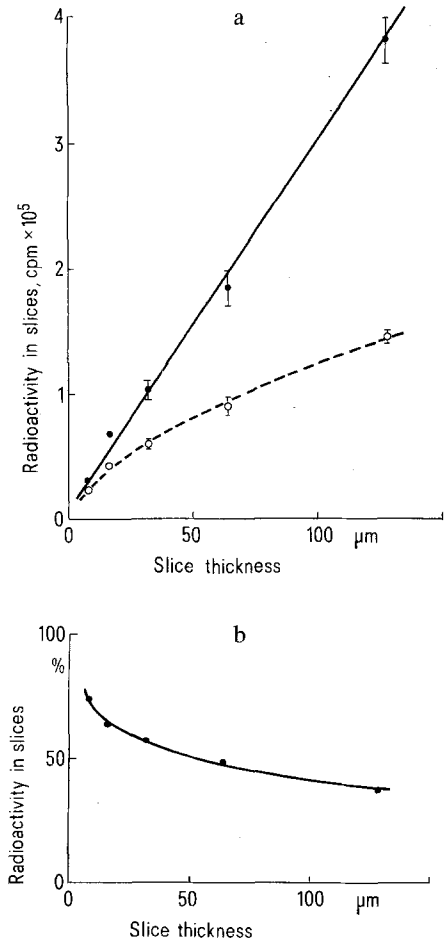

Fig. 2. *a* Radioactivity counts of the whole intact sections (●——●) and radioactivity of the same sections following their solubilization (○- - - -○) against their thickness; means of measurements ±SEM for 3–5 sections. *b* Ratios of the radioactivity levels before and after solubilization of the sections (in %) against their thickness.

Radioactivity of a number of brain structures after injection of ^{35}S-methionine into the left lateral brain ventricle; counts for section of 64 µm thickness through apertures of aluminium foil. Corrections on the basis of figure 2b

Structure	Counts (cpm)	Corrected values of the counts
Hippocampus	344	688
Medial thalamic nucleus	260	520
Plexus choroideus in the 3rd ventricle	178	356
Paraventricular nucleus, hypothalamus	37	-
Background	35	-

sections and their thickness is linear. However, the radioactivity of the nonsolubilized intact sections is not proportional to the increase in their thickness (figure 2a). Figure 2b shows that the ratio of 'surface' radioactivity of intact, nonsolubilized sections to that after their dissolving changes from about 75% for 8-µm thick sections to about 40% for sections of 124 µm thickness.

The data from the table can be corrected with the aid of figure 2b to yield the real values of radioactivity extent under the aperture in the foil. For sections of 64 µm thickness, the increment is in the order of 50% (table). This calibration curve is valid only for brain tissue, since other tissues obviously have their own characteristic absorption of radioactivity. However, the method itself is simple, rapid and reproducible, and may be applied for investigation of various tissues.

1 The financial support of the Israel Cancer Association is gratefully acknowledged.
2 Acknowledgment. We wish to thank Professor H. Nathan for his help and support and Professor J. Lenge for his constructive reading of the article.
3 K.B. Eik-Nes and K.R. Brizzee, Biochim. biophys. Acta *97*, 320 (1964).
4 A.W. Rogers, Techniques of autoradiography. Elsevier Publishing Comp., New York 1969.

A simple technique for testing the in vitro response of rabbit lymphocytes to PHA and allogeneic cells[1]

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Summary. Lymphocytes of rabbits can be separated from small quantities of heparinized whole blood using a simple density gradient of Ficoll-Ronpacon 1.09. This separation technique yields a pure suspension of viable cells allowing reproducible results from cultures stimulated either with PHA or allogeneic lymphocytes isolated by the same technique.

Histocompatibility studies and immunological function tests rely on reproducible lymphocyte cultures in animal models and in man. Numerous techniques for rabbit lymphocyte cultures have been described[2-6]. However, important differences in lymphocyte separation technique made a direct comparison impossible with methods used in human lymphocyte studies where reproducibility from laboratory to laboratory is good.

For our studies on bone marrow transplantation, we needed a simple test system for the rabbit allowing repeated cultures from the same animal, in order to assess histocompatibility by mixed lymphocyte culture (MLC).

We used a slight modification of a sedimentation gradient with Ficoll-Ronpacon employed routinely in human tests. Performing the sedimentation on a higher density gradient allowed lymphocyte cultures with reproducible stimulation by PHA and allogeneic cells with small amounts of peripheral blood obtained from living animals.

Materials and methods. 1. Animals. Normal outbred rabbits of 2 different strains, Burgundy and Dutch, were used as lymphocyte donors. Mixed lymphocyte cultures were performed between the 2 strains, which were supposed to be histo-incompatible.

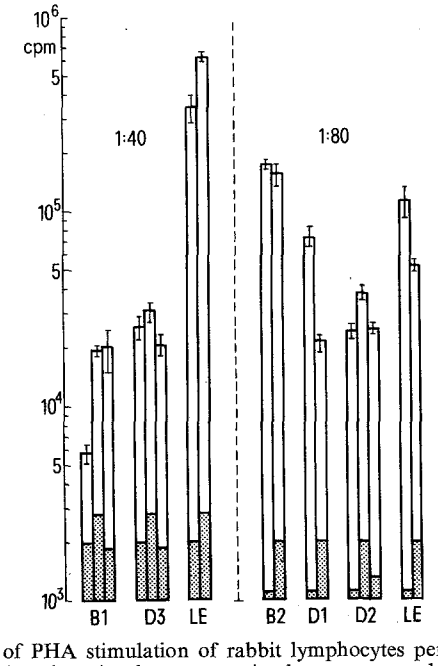
2. Lymphocyte separation. 8–10 ml of heparinized blood (1000 IU/10 ml Liquemin®) was drawn from the artery of the ear under sterile conditions and diluted with 10 ml phosphate buffered saline (PBS). 4 ml of diluted blood was layered on 2 ml Ficoll-Ronpacon with a density of 1.09 and

spun at 1000× g for 10 min (Rotanta-S Hettich Centrifuge). This density gradient of 1.09 gave the best yields of mononuclear cells (92%). The lymphocyte layer was aspirated, resuspended and washed twice in PBS. The lymphocytes were then resuspended in 5 ml minimal essential medium (MEM) with glutamin and penicillin/streptomycin, counted and adjusted by adequate dilution to a final concentration of 1.5×10^6 cells/ml.

3. Lymphocyte cultures. Cultures were performed on Microtiter Plates (Dynatech MA4) in quadruplicates. Each culture consisted of 1.5×10^5 cells/culture well. They were incubated at 37 °C, with 100% humidity and 5% CO_2 (Incubator National Appliance Company). No fresh culture medium was added during incubation. Mitogen-stimulated lymphocytes were incubated for 4 days and allogeneic-stimulated cells for 5 days prior to labelling. As DNA marker we used ^{3}H-thymidine: 50 µl, i.e. 40 µCi/well. For labelling, cells were incubated for 16 h under conditions described, and harvested on glass fibre filters (Minimash-Dynatech). Filters were put in scintillation counting solution and counted in a liquid scintillation counter. ^{3}H-thymidine uptake was expressed in cpm. Mean values of the quadruplicate results were calculated with their standard deviation. The counts were compared to background activity for PHA stimulation and to autologous stimulation for allogeneic cells stimulation.

4. Mitogen stimulation of rabbit lymphocytes. In a first experiment cells of 5 animals were exposed to PHA in decreasing concentrations from 1:1 (25 µl stock solution) to 1:400 (0.5 µl stock solution). Subsequent experiments were performed with PHA concentrations of 1:40 (0.6 µl) and 1:80 (0.3 µl) and compared with human lymphocytes under identical conditions.

5. Stimulation with allogeneic lymphocytes. For mixed lymphocyte cultures (MLC) irradiated lymphocytes of the opposite strain were used as stimulating cells. The concentration of these stimulating cells was adjusted to 3.0×10^6/ml. They were irradiated with 3500 rads (air dose) at a dose rate of 500 rads/min. (Linac, Linear accelerator). Autologous stimulating cells served as controls.



Results of PHA stimulation of rabbit lymphocytes performed on consecutive days in the same animals as compared to normal human lymphocytes. Results are expressed in cpm (±1 SD). LE: normal human lymphocytes, B: Burgundy rabbit lymphocytes, D: Dutch rabbit lymphocytes. Dotted area represents background counts.

Table 1. Results of stimulation of rabbit lymphocytes with various PHA concentrations. Results are expressed in cpm (±1 SD), compared to background cpm. B: Burgundy rabbits, D: Dutch rabbits

B 1	B 2	B 3	D 1	D 2	PHA
17,799 (6,012)	2,359 (695)	20,412 (1,052)	13,592 (1,720)	10,684 (1,070)	Stock solution (25 µl)
6,920 (1,460)	2,940 (999)	37,312 (3,785)	19,440 (1,991)	-	1:4 (6.25 µl)
12,055 (695)	4,708 (1,071)	23,680 (4,172)	30,699 (3,993)	5,377 (990)	1:8 (3.1 µl)
14,342 (5,530)	61,444 (2,731)	33,910 (994)	13,461 (703)	15,603 (2,325)	1:20 (1.25 µl)
20,865 (7,820)	157,918 (32,321)	57,446 (3,970)	47,974 (7,670)	33,562 (4,404)	1:40 (0.625 µl)
45,004 (3,290)	151,485 (12,580)	44,113 (4,270)	14,401 (1,733)	46,619 (4,362)	1:80 (0.312 µl)
-	42,195 (3,774)	7,517 (2,180)	5,494 (427)	-	1:400 (0.05 µl)
1,844 (342)	689 (150)	2,103 (295)	2,103 (295)	1,844 (342)	Background

cpm

Table 2. Results of the allogeneic stimulation of rabbit lymphocytes. The results are expressed in cpm (±1 SD). The stimulation index (I) is calculated by: cpm allogeneic stimulation: cpm autologous stimulation. B: Burgundy rabbit lymphocytes, D: Dutch rabbit lymphocytes

cpm	B 1	D 1
B 1	6,027± 902	8,045± 397 I=1.3
D 1	32,637±2,910 I=3.0	10,876± 712

1

cpm	B 2	D 2
B 2	6,497±1,000	25,352±3,500 I=3.9
D 2	25,504±6,780 I=4.0	6,307±1,630

2

cpm	B 3	D 3
B 3	7,248± 935	28,639±6,510 I=3.9
D 3	54,484±2,450 I=5.0	10,876± 712

3

cpm	B 4	D 4
B 4	4,402± 640	10,736±2,250 I=2.4
D 4	14,529±1,610 I=1.9	7,712± 346

4

Results. 1. About 20×10^6 cells (range $15\text{–}28 \times 10^6$) could be collected from 8 ml whole blood with this technique. The cell suspension contained 92% (range 90.5–93%) lymphocytes, 3.75% (range 2.5–5%) monocytes and 4.25% (range

3.5–5.5%) granulocytes. Viability assessed by dye exclusion was over 95%. 2. The response of rabbit lymphocytes to varying concentrations of PHA are summarized in table 1. Even if the response varied from animal to animal, a concentration of PHA of 1:40 or 1:80 was found to be optimal for rabbit lymphocytes of both strains examined under the culture conditions described. These concentrations were used in the further experiments of this study. 3. Results of PHA stimulation of rabbit lymphocytes and of human lymphocytes cultured under the same conditions are presented in the figure. Stimulation varies from animal to animal, but the stimulation of rabbit lymphocytes is consistently better at lower concentrations compared to human cells. 4. Results of the allogeneic stimulation of rabbit lymphocytes are summarized in table 2. It shows clearly that rabbit lymphocytes can be stimulated by allogeneic cells. The responses observed with allogeneic cells is consistently lower than with mitogen (PHA) which is also well known from other animals. The response is also lower than usually observed with human lymphocytes in a similar system.

Discussion. The results presented here demonstrate that rabbit lymphocytes can be reproducibly separated from small quantities of whole blood on a 1.09 Ficoll-Ronpacon density gradient. This sedimentation technique yields a quite pure suspension of viable lymphocytes (90–93%) with about 4% monocytes and a contamination of less than 6% granulocytes. The number of cells harvested is also fairly reproducible, about 20×10^6 cells out of 8 ml whole blood. This allows various tests from a single sample of blood. The quality of the cells permits reproducible cultures either for stimulation with PHA or with allogeneic cells. Results are comparable to those published by other authors using other techniques[5,6].

As recently demonstrated by Bell[12], the feature of rabbit T-lymphocytes appear to be quite similar to those of human T-cells. T-cells of the rabbit are PHA responsive. Rabbit lymphocytes stimulated by PHA show a variation from animal to animal, as expected from other experiments and from comparison with the human system. This appears to make functional tests of T-lymphocytes of the rabbit possible which are important for studies on allogeneic bone marrow transplantation. The amount of PHA needed for optimal stimulation of rabbit lymphocytes appears to be quite similar to the human one[10], about 0.5 µl–0.25 µl of stock solution per culture.

Compared to human controls, the response of rabbits is usually lower, despite identical cell concentrations and culture conditions. This has already been shown by other investigators and is also known from other species[6,8,9]. The reason for this is not yet understood. This difference appears particularly important in allogeneic lymphocyte stimulation where it may be difficult to compare results of animals and man[10,11]. The fact that the stimulation with allogeneic lymphocytes is lower than PHA stimulation of T-cells, is also well known in man. Here again the similarity of rabbit T-lymphocytes is evident. It is well known[13] that about 40% of human T-lymphocytes will be stimulated by PHA whereas less than 5% respond to PPD, even after sensitization, or to allogeneic cells. This may explain the difference in H_3-thymidine incorporation.

In conclusion: We have shown that stimulation of rabbit lymphocytes by mitogens or allogeneic cells is possible on cells separated on a Ficoll-Ronpacon density gradient of 1.09 from small quantities of peripheral blood. Culture results are fairly reproducible and comparable to those previously published using different techniques. This separation technique allows repeated tests of rabbit T-lymphocyte function, as well as histocompatibility testing using mixed lymphocyte cultures.

1 Supported by the Swiss Science Foundation No. 3.890-0.77.
2 H. Matej, Arch. Immun. Ther. Exp. *18*, 315 (1970).
3 C. Bell and S. Dray, Cell. Immun. *6*, 375 (1973).
4 R.G. Tissot and C. Cohen, Tissue Antigens *2*, 267 (1972).
5 R.G. Tissot and C. Cohen, Transplantation *18*, 142 (1974).
6 V.C. Miggiano, M. Tommaso, I. Birgen and M. Nabholz, Tissue Antigens *5*, 173 (1975).
7 P.D. Utsinger, W.J. Yount, J.G. Fallon, M.J. Loque, R. Fuller and D. Elliot, Blood *49*, 33 (1977).
8 E. Gluckman, M.L. Schweder, R. Storb, H. Goselink, S. Johnson, I.C. Graham, G. Pretorius and E.D. Thomas, Transplantation *19*, 36 (1975).
9 R. Storb, P. Weiden, T. Schweder, C. Graham, K.G. Lerner and E.D. Thomas, Transplantation *21*, 299 (1976).
10 R.J. Hartzmann, M. Segall, M.L. Bach and F. Bach, Transplantation *11*, 268 (1971).
11 M. Shapira and M. Jeannet, Tissue Antigens *4*, 178 (1974).
12 C. Bell and H. Wigzell, Eur. J. Immun. *7*, 726 (1977).
13 E. Herva and K. Kiviniitty, Strahlentherapie *149*, 504 (1975).

Interphase studies with a simplified method of silver staining of nucleoli

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Summary. A simple silver staining method is presented providing a rapid and reliable technique for the selective staining of nuclear structures synthesizing ribosomal RNA (18S and 28S RNA).

Many techniques for the selective staining of nucleoli and RNA-rich structures are available. Their application depends on the use either of basic dyes[2–4], or basic dyes plus cationic surfactant[5], or silver-salt solutions[6,7]. More recently developed silver staining methods[8–10] have been widely used to detect nucleolar organizing regions (NORs) in the karyotypes of man and other organisms. There is evidence from studies in somatic cell hybrids that those NORs are stained by silver which were actively engaged in the synthesis of rRNA during the preceding interphase[11,12]. Furthermore, the silver staining patterns obtained from preparations of early embryonic stages[13], and those from stages of male gametogenesis[14], provide evidence that silver staining reflects the function of ribosomal genes rather than their mere presence.

However, with the exception of these latter studies there are very few applications of this method to interphase cells. In a study about the nature of Ag-staining, Schwarzacher et al.[15] made it most plausible that the Ag-granules are mainly associated with the fibrillar component of the nucleolus. A positive direct correlation between the amount of silver precipitates over the nucleolar regions and the rate of rRNA synthesis was clearly demonstrated by Hofgärtner[16].