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Separation of Thymus-derived and Marrow-derived Rat Lymphocytes on Glass Bead Columns

S. V. HUNT

Medical Research Council Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford University, Oxford, England, OX1 3RE

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Summary. Rat thoracic duct lymphocytes were passed through columns of fine siliconed glass beads according to the method of Shortman (1966). On the basis of the degree of labelling with uridine *in vitro* and of surface antigenic markers it was shown that marrow-derived small lymphocytes were preferentially retained on the columns, causing the concentration of thymus-derived small lymphocytes in the filtered cells. Filtered cells retained normal graft-versus-host (GVH) activity. The proportion of thymus-derived small lymphocytes in normal rat lymph was estimated at a maximum of 63–87 per cent. Previous reports on the immunocompetence of filtered cells may readily be explained by these findings.

INTRODUCTION

In the course of experiments concerning the properties of rat thoracic duct lymphocytes (TDL) before and after filtration through glass bead columns (Shortman, 1966) it was suspected that in addition to removing large lymphocytes the filtration procedure also discriminated between subpopulations of small lymphocytes (Hunt, Ellis and Gowans, 1972). This communication substantiates the idea that marrow-derived small lymphocytes (B lymphocytes) are retarded during passage of rat TDL through columns of siliconed glass beads, causing the enrichment of thymus-derived (T) lymphocytes in the filtrate. The immunological behaviour of the filtered cells is readily explicable in the light of these results.

MATERIALS AND METHODS

Animals and treatment of cells

Young adult inbred hooded (HO) or agouti (DA) rats of either sex were used. TDL from the first over-night collection were obtained (Gowans and Knight, 1964) washed once in DAB/2 per cent FCS (DAB, Dulbecco's A+B, Oxoid Ltd; FCS, Foetal Calf Serum) at 4° and labelled by incubation for 1–2 hours with Uridine-5-^{3}H (5 μCi/ml) or Uridine-(U)-^{14}C (2 μCi/ml) (Howard, 1972). After three washes at 4° in DAB/2 per cent FCS the cells were resuspended in DAB/10 per cent FCS for fractionation. Estimation of the specific radioactivity of samples before and after separation and auto-radiographic procedures have been described (Howard, Hunt and Gowans, 1972).

A chimaeric rat with alloantigenically marked T lymphocytes was an HO rat prepared by thymectomy, irradiation and reconstitution with 10^{7} lymphocyte-depleted bone-marrow

cells ('B rat') (Howard, 1972); 5 weeks later, it received 10^9 thymocytes from young (HO × DA) F_1 rats. TDL were collected 16 weeks later still.

Columns

A detailed account of the fractionation prodecure has been published (Hunt, Ellis and Gowans, 1972). Briefly, $0{\cdot}6–5 \times 10^8$ TDL at 10^8/ml in DAB/10 per cent FCS were run at 4° slowly through short (about 6 cm) columns of siliconed glass beads, 53–74 μm diameter, following the method of Shortman (1966). If the number of cells passing through the column exceeded 15 per cent of those introduced, the filtered cells were passed through a second smaller column. 'Passed' denotes the fraction obtained from the bottom of the last column. 'Recovered' denotes the fraction obtained by pumping eluant upwards through the first column after fractionation.

Cytotoxic analysis

Detection of lymphocytes bearing DA alloantigens followed the procedure of Scott and Howard (1972), using an HO-anti-DA alloantiserum: reagent volumes were 25 instead of 50 μl. The results are shown as per cent cells killed specifically by the antiserum after allowing for the small percentage (<4 per cent) found dead with normal serum as control.

The antiserum specific for B lymphocytes ('anti-B') was a generous gift of Dr J. C. Howard. It was raised by repeated immunization of a rabbit with TDL from B rats and was thoroughly absorbed with rat thymocytes. Its specificity will be accredited elsewhere (Howard and Scott, to be published). Results are shown as per cent cells not killed by the serum: the figures tend to overestimate proportions of T small lymphocytes since large lymphocytes are also not killed by the serum.

RESULTS

The glass bead columns devised by Shortman (1966) were originally intended to provide a simple method for the removal of large lymphocytes from lymphocyte suspensions: the

TABLE 1

FRACTIONATION OF NORMAL RAT TDL ON GLASS BEAD COLUMNS

Expt No.	^{3}H- or ^{14}C-uridine specific activity (cpm/10^6 cells)			
	Before fractionation	'Passed'	(Ratio*)	'Recovered'
1	8256	11763	(0·70)	8330
2	15774	24985	(0·63)	N.D.
3	6695	9316	(0·72)	5288
4	26261	30213	(0·87)	20662

In experiment 4, fractions were also tested for cytotoxicity with anti-B antiserum. Allowing for per cent found dead with normal serum (3, 7, 2 per cent respectively) the per cent killed by anti-B were: Before fractionation, 40 per cent; 'passed', 0·5 per cent; 'recovered', 59 per cent.

* Ratio $= \frac{\text{Specific activity before fractionation}}{\text{Specific activity 'passed'}}$.

N.D. = Not done.

purity of the small lymphocytes in 'passed' fractions has been documented (Shortman, 1966; Shortman *et al.*, 1971; Lewis, Mitchell and Nossal, 1969). Their ability to distinguish sub-populations of small lymphocytes was shown as follows.

TDL from normal HO rats were labelled *in vitro* with radioactive uridine and the specific activities of the fractions were measured before and after separation on columns of small glass beads at 4°. Table 1 shows that in each of four experiments the specific radioactivity increased in the 'passed' fraction, the opposite result to that expected if the action of the column were confined solely to the removal of large lymphocytes, which take up relatively large quantities of uridine *in vitro* (Howard, Hunt and Gowans, 1972). The finding of an enriched activity (also illustrated by grain count distributions in autoradiographs, Fig. 1) is explicable in the light of the demonstration that there exists in the thoracic duct

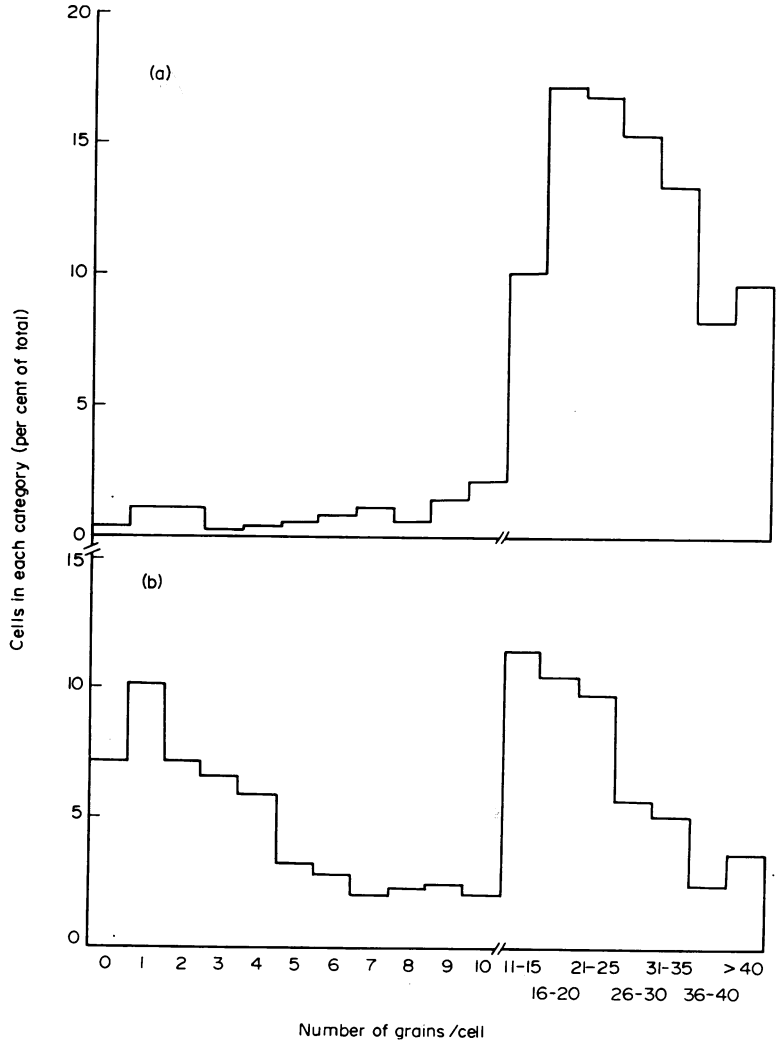

FIG. 1. Grain count distributions over autoradiographs of (a) 'passed' and (b) unfractionated normal TDL labelled with ^{3}H-uridine (experiment 2, Table 1). Exposure, 3 days. Only small lymphocytes were counted (702 on each smear). Lightly-labelling cells are removed by passage through the column.

lymph of normal rats a sub-population of small lymphocytes which labels heavily with uridine and is thymus-derived (Howard, Hunt and Gowans, 1972), in contrast to the lightly-labelling B small lymphocyte sub-population. The columns must selectively trap B small lymphocytes.

This conclusion was confirmed in an experiment using a heterologous antiserum specific for B small lymphocytes. The percentage of cells not killed by this anti-B antiserum and complement rose from 60 in the unfractionated TDL to 99·5 in the 'passed' fraction (Table 1). A corresponding decrease of T lymphocytes in the 'recovered' fraction was noted.

That the cells able to pass through the columns were thymus-derived was formally demonstrated in an experiment with TDL from a chimaeric rat, in which the T lymphocytes were derived from an inoculum of semi-allogeneic thymocytes carrying a distinctive surface alloantigenic marker (from (HO × DA)F_1 hybrids, see Materials and Methods). Cytotoxic analysis with an HO-anti-DA alloantiserum again showed enrichment of thymus-derived lymphocytes in the 'passed' fraction, and their corresponding depletion in the 'recovered' fraction (Table 2). The proportions agree well with those found by

TABLE 2

FRACTIONATION OF TDL FROM CHIMAERIC RAT BEARING ALLOANTIGENICALLY-MARKED T CELLS

	Per cent T cells by:			
	Anti-DA*	Anti-B†	^{14}C-Uridine cpm/10^6 cells	Ratio‡
Before fractionation	28	33	12686	
'Passed'	63	65	20337	0·62
'Recovered'	21	33	12021	

59×10^6 ^{14}C-uridine-labelled TDL from the chimaeric rat described in Materials and Methods were passed through two columns in succession. The second 'passed' fraction contained $11{\cdot}5 \times 10^6$ cells (19 per cent): 47×10^6 cells (80 per cent) were 'recovered' by upwards elution from the first column.

*Per cent killed by alloantiserum allowing for dead cells in controls with normal serum (always < 4 per cent).

†Per cent alive, allowing for dead cells in controls (< 4 per cent).

‡ See Table 1.

treatment with anti-B serum and complement, when allowance is made for the failure of anti-B serum to kill large lymphocytes. The degree of purification of T cells was less good than in experiment 4 with normal TDL (Table 1), presumably because unfractionated chimaeric TDL contained more B lymphocytes (72 against 40 per cent).

GRAFT-VERSUS-HOST (GVH) ACTIVITY

After fractionation of normal DA TDL the GVH activity of 'passed' cells (measured by the popliteal lymph node weight assay of Ford, Burr and Simonsen (1970)) was at least equal to that of unfractionated TDL and was perhaps slightly enhanced (Fig. 2). This finding agrees with the good evidence in the mouse that GVH activity is a property of T lymphocytes (Cantor, 1972).

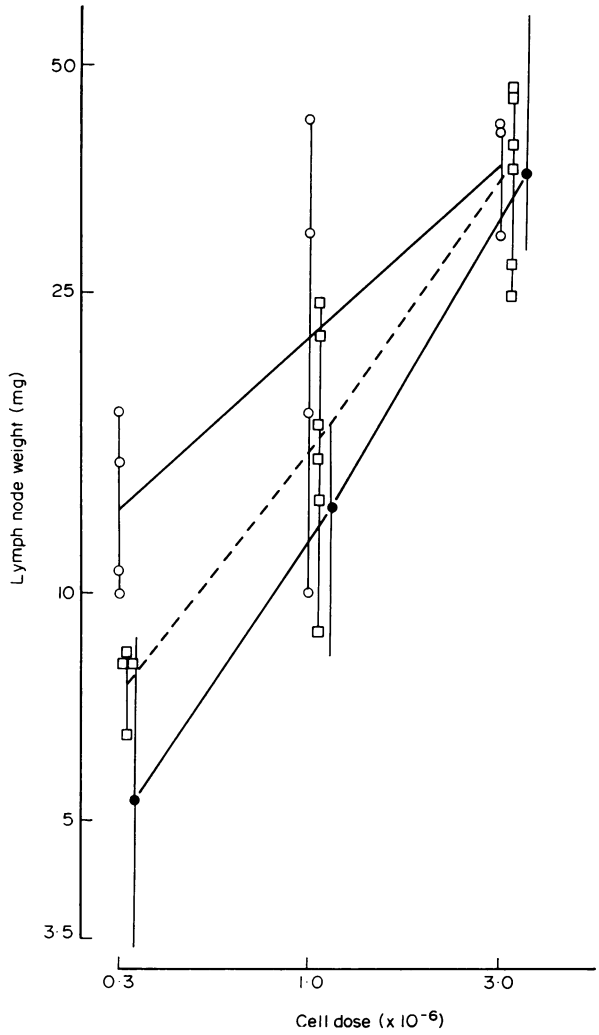

FIG. 2. GVH activity of normal TDL before and after fractionation (DA→(AO × DA)F_1: strong histocompatibility difference Ag-B4→Ag-B2). Popliteal lymph node weight assay, 6 days after injection into hind footpad. Log scales. Individual node weights shown for (○) 'passed' and (□) 'recovered' fractions. Mean ± range for nine nodes at each cell dose shown for (●) fresh TDL.
'Passed' cells are at least as reactive as cells before fractionation.

DISCUSSION

The experiments described here show that columns of fine glass beads can preferentially remove the marrow-derived sub-population of small lymphocytes from rat TDL. They confirm the suspicions of Lewis, Mitchell and Nossal (1969) and of Hunt, Ellis and Gowans (1972) aroused by the failure of column-purified small lymphocytes efficiently to confer primary or secondary humoral immune responsiveness on irradiated recipients in marked contrast to the normal competence of small lymphocytes prepared by other means. This failure is readily comprehensible in the light of the demonstration here that B lympho-

cytes (i.e. precursors of antibody-forming cells) are depleted in column-purified small lymphocytes. The inability of Lewis, Mitchell and Nossal (1969) to restore the immune competence of 'passed' cells by addition of bone marrow cells must be attributed to the use of an insufficient dose of cells from an organ not especially rich in mature B lymphocytes. The finding that the GVH activity of 'passed' cells is normal (Shortman and Szenberg, 1969, and confirmed here (Fig. 2)) agrees with the notion that this reactivity is a property of T cells.

The characteristic responsible for the trapping of B lymphocytes cannot be cell size, since both B and T small lymphocytes are of approximately similar diameters in smears or judged by the Coulter Counter; it must be presumed instead that surface adherence differs in the sub-populations. There are repeated hints in the literature that B small lymphocytes are stickier than T (Bianco, Patrick and Nussenzweig, 1970; Tan and Gordon, 1971; Rosenthal, Davie, Rosenstreich and Blake, 1972; Rosenstreich, Shevach, Green and Rosenthal, 1972) and that this difference in stickiness persists or may be magnified after subsequent differentiation (e.g. to antibody-producing cells, Plotz and Talal, 1967; Salerno and Pontieri, 1969; or to activated T cells, Hogg and Greaves, 1972).

The figures for the specific activities of uridine uptake (Table 1) may be used to deduce a maximum estimate of the proportion of T small lymphocytes in rat TDL. The specific activity (U) of unfractionated cells is the sum, over all component populations, of the products of the specific activity and the proportion of each population in normal TDL. Therefore, omitting the contributions of large lymphocytes and B small lymphocytes,

$$U \geqslant \text{specific activity of T cells} \times \text{proportion of T cells}$$

$$\text{i.e. proportion of T cells} \leqslant \frac{U}{\text{specific activity of T cells}}$$

and, since specific activity of T cells > specific activity of 'passed' fraction which may contain a contamination due to low activity (B) cells,

$$\text{proportion of T cells} \leqslant \frac{\text{specific activity unfractionated cells}}{\text{specific activity 'passed' fraction}}$$

(see Ratio, Table 1).

Hence maximum estimates of the proportions of T small lymphocytes range from 87 to 63 per cent in different experiments. This argument ignores the contributions of large lymphocytes and B lymphocytes to the uridine uptake and the contamination that may sometimes be expected by B lymphocytes in the 'passed' fraction. If they were taken into account, the estimates would have to be revised downwards. The values are lower than those usually quoted for mouse TDL (Raff, 1971; Miller and Sprent, 1971) and serve to re-emphasize that not all lymphocytes of the recirculating pool are thymus-derived (Howard, Hunt and Gowans, 1972). On the simplest model, which assumes only T and B subpopulations in thoracic duct lymph, the minimum proportion of marrow-derived lymphocytes ranges from about 15 to 35 per cent.

It should be stressed that, as presently designed, the columns yield poor cell recoveries and that these can vary from one experiment to the next even after some experience of the technique. However, glass bead column filtration provides a simple way of removing large lymphocytes, damaged cells and B lymphocytes of the rat in a single operation; and the purification of T lymphocytes can sometimes be very effective (experiment 4, Table 1).

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