Immunology, 1969, **17**, 955.

Subpopulations of Rat and Mouse Thoracic Duct Small Lymphocytes in the *Salmonella* Flagellar Antigen System*

HEATHER LEWIS, JUDITH MITCHELL AND G. J. V. NOSSAL

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, *Australia*

(*Received* 22*nd April* 1969)

SUMMARY

Summary. The purpose of the present experiments was to determine whether populations of lymphocytes from mouse or rat thoracic duct lymph deprived of the larger, dividing cells (large and medium lymphocytes) could still transfer adequate primary or secondary adoptive immune responses using syngeneic irradiated hosts. The transfer system used involved the antigen polymerized flagellin from *Salmonella adelaide* and assay of anti-H antibody levels in the recipients. Two methods of preparation of 'small lymphocytes' were used, namely the glass bead column filtration method of Shortman and the agitated culture method of Gowans and Uhr (1966). Both of these yield lymphocyte fractions essentially free of dividing cells.

Unfractionated thoracic duct lymphocytes gave satisfactory adoptive immune responses in both species, primed cells being more effective than normal cells.

In both species and for both primary and secondary immune responses, the Shortman-column fractionated small lymphocytes (col-SL) were grossly impaired in their capacity to transfer adoptive responses. In contrast, the Gowans-type post-incubation small lymphocytes (inc-SL) gave rather better adoptive immune responses. Gowans and Uhr (1966) had found some enhancement of reactivity of inc-SL in comparison with normal lymph cells in another transfer system. We, however, found a mild impairment with unstimulated mouse and rat inc-SL and primed rat inc-SL and a slightly more marked impairment with primed mouse inc-SL.

We have confirmed the finding of Gowans and Uhr (1966) that lymphocyte populations free of dividing cells can mount an adoptive antibody response, showing that a proportion, at least, of the antigen-reactive cells can be described as 'small lymphocytes'. The study also revealed the functional heterogeneity of morphologically similar lymphocytes, in that col-SL, morphologically the same as inc-SL, are functionally quite different; they appear to contain very few antigen-reactive cells. In the *Salmonella* system, therefore, the antigen-reactive thoracic duct cells can be described as lymphocytes which are fairly resistant to agitated culture but which fail to pass through Shortman columns.

* This is publication No. 1293 from The Walter & Eliza Hall Institute.

At present, we are reluctant to state that column fractionated small lymphocytes were deficient in one class of cell necessary for some cell–cell interaction, as we failed to reconstruct the population's immune potential by addition of various other cells. However, particularly in view of results coming forward in other systems, this possibility can by no means be excluded.

INTRODUCTION

While it is clear that pure populations of lymphocytes possess the capacity to confer antibody-forming potential to immunologically non-reactive host animals, our knowledge of the detailed properties of these specific cells which can respond to antigen is fragmentary. In particular, it is still not possible to obtain antigen-reactive lymphocytes in anything approaching a pure suspension, and this impedes detailed study of the inductive mechanisms involved in antibody formation. Presumably such purification, or even significant enrichment of antigen-reactive cells will be possible only after careful characterization of all their properties, such as size (Shortman, 1966; Gowans and Uhr, 1966) electrophoretic mobility and volume (Ruhenstroth-Bauer and Lücke-Huhle, 1968) buoyant density (Peterson and Evans, 1967; Shortman, 1968a) and organ derivation (Miller and Mitchell, 1968).

In the present paper we report studies relevant to the question of whether small lymphocytes free of all large, dividing cells can mount adoptive immune responses to a pure, strongly immunogenic protein antigen, *Salmonella* flagellin. As background to the study, we have extensive evidence from the work of Gowans (Gowans and McGregor, 1965; Gowans and Uhr, 1966) which suggests that long-lived, recirculating small lymphocytes carry immunological information and can transform into antibody-forming cells on appropriate stimulation. Pure small lymphocytes can also effect graft-*versus*-host reactions (Gowans, 1962; Shortman and Szenberg, 1969) and can induce the formation of small foci of cells forming antibody against sheep erythrocytes in the spleens of lethally-irradiated antigenically stimulated mice (Mitchell and Miller, 1968; Nossal, Shortman, Miller, Mitchell and Haskill, 1967). It would thus be tempting to assume that all immune phenomena involve activation and clonal expansion of long-lived small lymphocytes.

This simple and attractive view has been challenged on the basis of two findings. First, pulse-labelling experiments with [^{3}H]thymidine performed by our group (Nossal and Mäkelä, 1962; Mitchell, McDonald and Nossal, 1963) suggested that a rapidly-cycling cell might be the precursor of cells forming anti-*Salmonella* antibodies in the rat. These studies were complicated by insuperable problems of isotope reutilization and therefore could not be regarded as definitive. Secondly, an extensive series of studies in the mouse using erythrocytes as antigen has suggested that in this system, *optimal* antibody production involves a cell–cell interaction (or collaboration) between thymus-derived, recirculating lymphocytes and bone-marrow derived (possibly more rapidly-cycling) lymphocytes, the latter acting as the actual precursors of the antibody-forming cell (Miller and Mitchell, 1968; Nossal, Cunningham, Mitchell and Miller, 1968).

We report findings which complicate the picture still further. We have used two current methods for the preparation of 'pure' small lymphocyte suspensions (Gowans, 1962; Shortman, 1966) and have tested their potency both in primary and secondary adoptive responses in two species, rat and mouse.

MATERIALS AND METHODS

Animals

Inbred male DA rats, originally obtained from the Wistar Institute, Philadelphia and maintained at the Hall Institute were used at 10–16 weeks of age as thoracic duct lymphocyte donors or at 6–8 weeks as recipients. They were fed on 'Barastoc' dog cubes and tap water. Recipient rats were kept two per box in mouse cages with easy access to food and water.

Inbred mice of the CBAT6T6 strain, originally obtained through the courtesy of Dr C. E. Ford from the Radiobiological Research Establishment, Harwell and maintained at the Hall Institute were used at the same ages as were the rats and were fed similarly. Mice of both sexes were used but the sexes were not mixed within one experiment.

Primary immunization of lymphocyte donors

Antigenically stimulated cell populations were obtained from animals which had been injected 4–8 weeks previously with 10 μg *S. adelaide* polymerized flagellin (Ada, Nossal, Pye and Abbot, 1964). This dose had been spread over five sites, subcutaneously into each footpad and intraperitoneally, and no adjuvant was used.

Collection of thoracic duct lymph

The thoracic ducts of donor rats were cannulated under nembutal anaesthesia using the method of Williams (1966). For the cannulation of mice we are indebted to Dr G. Mitchell who used a modification of the method originally described by Boak and Woodruff (1965).

Cells from individual animals were collected into Dulbecco's medium (Oxoid Ltd, U.K.) containing 10 per cent inactivated foetal calf serum (FCS, Commonwealth Serum Laboratories, Melbourne), 100 units/ml penicillin and 2–10 units/ml preservative-free heparin (Boots Ltd) in covered, sterile, siliconized tubes. The collecting tubes were kept in ice during the entire collection period from 4 to 24 hours after cannulation.

At the end of a collection period the cells were pooled and centrifuged at 1000 ***g*** for 7 minutes in the cold. They were resuspended in a small volume of medium appropriate for either column fractionation or agitated culture.

Column fractionation of lymph cells (Shortman, 1966)

After centrifugation the cells were resuspended in Dulbecco's medium (containing 10 per cent FCS and 100 units/ml penicillin) and their numbers counted. Ten per cent of this yield was removed and stored at 4° until the remaining cells had been passed through the column. These cells are described as pre column thoracic duct lymphocytes or pre-col-TDL.

The cells for column separation were run through a large (1·5–3 cm diameter), cold (4°) column of siliconized glass beads (53–74 μ diameter) at a rate between 2 and 10 seconds per drop. If the resultant yield was greater than 15 per cent the cells were concentrated by centrifugation and passed through a smaller fresh column. A final composite yield containing 5–15 per cent of the initial TDL was used as the inoculum of column-fractionated thoracic duct small lymphocytes or col-SL.

Estimates of the viabilities of pre-col-TDL and col-SL were made using eosin dye-exclusion and [^{3}H]uridine uptake as described below. Both methods indicated 90–98 per cent viability immediately before injection.

Preparations of col-SL were checked for the exclusion of large and medium cells by comparing them with pre-column cells for ability to incorporate [^{3}H]thymidine *in vitro* as described below.

Preparation of pure small lymphocytes by agitated culture

The method of Gowans (1962) was applied to thoracic duct lymphocytes of both rats and mice. After collection and centrifugation the cells were suspended in medium 199 (Commonwealth Serum Laboratories, Melbourne) containing 100 units penicillin and 100 μg streptomycin/ml, 20 per cent v/v phosphate buffered saline, pH 7·3 and 1 per cent inactivated serum of the species under study.

Two-thirds of the cells were suspended at a concentration of 50–100$\times 10^6$ cells/ml and incubated in 25-ml stoppered flasks. Each flask contained 5–8 ml and was incubated at 37° with constant slow rocking. After 24 hours, cells from all flasks were pooled and used as 'incubated thoracic duct small lymphocytes' or inc-SL. The remaining one-third of the cells were stored at 4° for 24 hours and were termed 'stored' cells.

In some experiments animals were injected with untreated fresh cells to provide a comparison between fresh and stored untreated lymphocytes. The time relationships between the irradiation of recipient rats and the injections of both cells and antigen were the same as in the main experiments.

Total cell viability estimations were made immediately prior to injection and the dose of cells injected was corrected according to viability. We found that both storage and agitated culture reduced the viability, to 80–95 per cent viable after storage, to 30–40 per cent after shaking rat cells or to 55–68 per cent after shaking mouse cells.

Measurement of cell function by the uptake of tritiated nucleotides

In all experiments cell samples were taken just before injection to detect any metabolic differences between treated and control cells apparent on *in vitro* uptake of nucleic acid precursors. The methods have been described in detail (Mitchell, 1964). Cells were suspended at a concentration of 1–5$\times 10^6$/ml and incubated for 1 hour at 37° with 20 μc of [^{3}H]uridine. They were then concentrated by centrifugation, resuspended in FCS and smeared on gelatin-coated slides for autoradiography using Kodak NTB-2 liquid emulsion. Exposure times of 14–60 days were allowed to detect a maximal proportion of labelled cells, the percentage of which was counted. Slides exposed for shorter periods (1–7 days) were used to distinguish the morphology of the heavily labelled cells.

The uptake of [^{3}H]thymidine, present at a concentration of 1–2 μc/ml was studied by the same method.

Recipients

In the experiments using rats, recipient animals were bled 1–4 days prior to cell transfer to ensure the absence of antibody. They were irradiated 2–6 hours prior to cell injection. The rats were given a whole body dose of 700 rads under maximum backscatter conditions using a Philips RT 250 machine operating at 120 rads/min, 250 kV, half-value layer (HVL) 0·8 mm Cu at a dose rate of 125 rads/min. Opposing fields were used, half the total dose time administered to one side of a Perspex rat container and the remainder to the opposite side. The focal skin distance was 50 cm. Some littermates were kept as non-irradiated controls for the effectiveness of the antigen preparation.

For mice the recipients were irradiated with 800 rads and otherwise treated as the rats.

Cells were injected intravenously into the tail veins in both species in volumes of 0·5 ml for rats and 0·3–0·6 ml for mice.

Antigenic challenge with 10 μg *S. adelaide* polymerized flagellin was given intravenously 1 day after the injection of cells. The animals were bled from the tail vein 7 days after antigenic challenge. In some cases earlier bleeds were taken if the animals were sick and those which survived until 14 days were again bled and killed. Where antigen was not given, the animals were bled at the corresponding time after cell injection.

Serum antibody titrations were performed using the *Salmonella* immobilization technique described by Ada *et al.* (1964). As far as possible sera were not frozen before titration.

RESULTS

A. RAT THORACIC DUCT LYMPHOCYTES

1. *Potency of column-fractionated small lymphocytes in adoptive immune responses*

We compared the efficacy of col-SL and pre-col-TDL from antigenically primed rats in a secondary adoptive immune response (Fig. 1). Five million pre-col-TDL gave uniform and reproducible antibody titres in the recipients after appropriate re-stimulation, and 10×10^6 cells conferred an immune reactivity approximately comparable to that of a normal intact rat. As expected, rats receiving pre-col-TDL but *not* an antigenic stimulus did not manufacture detectable antibody. The col-SL population was obviously grossly depleted in its capacity to confer an adoptive secondary response. Antibody titres of recipients were negligible even with the highest dose of cells used, namely 20×10^6. Nevertheless, it is possible that if it had been practicable to use numbers of column fractionated

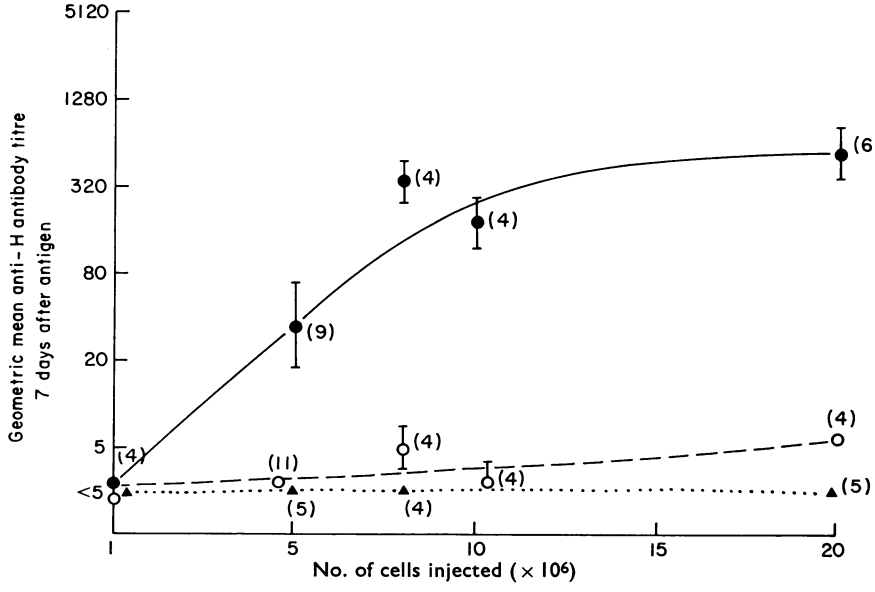

FIG. 1. The effect of Shortman column fractionation on primed rat thoracic duct lymphocytes transferred to irradiated syngeneic hosts. ○, Column fractionated cells (col-SL) in recipients challenged with antigen 1 day later; ●, unfractionated cells (TDL) in recipients challenged with antigen 1 day later; ▲, unfractionated TDL in recipients not challenged with antigen. Numbers in parentheses indicate the number of animals at each point. Vertical bars represent standard deviation.

small lymphocytes comparable to those used by Gowans and Uhr (1966), e.g. 200×10^6, significant titres would have developed. However, over the cell dose-range studied by us it appears that col-SL are depleted by a factor approaching 100 in their content of antigen-reactive cells as compared with pre-col-TDL.

Certain important controls are given in Table 1, which shows that the antigen alone never induced a significant immune response in irradiated rats, but always adequately stimulated normal animals. Essentially similar findings applied to mice.

TABLE 1

ANTIGEN AND IRRADIATION CONTROLS, POOLED FROM ALL EXPERIMENTS ON RATS

Treatment	No. of rats	Geometric mean titre 7 days after antigen	Range
X-irradiated, antigen 24 hours later	25	<5	All <5
Normal, antigen alone	17	201	15–4800

It seemed possible that cell–cell interactions, so important in the response of mice to sheep erythrocytes (Miller and Mitchell, 1968) could also be at work in the *Salmonella* system, particularly as this has been shown to be thymus-dependent in the rat (Lind, 1969). Accordingly, we sought to determine whether column fractionated SL were deficient *not* in antigen-reactive cells but in some non-specific, interacting cell. We performed a reconstruction experiment in which col-SL from a primed rat was mixed with either TDL or bone marrow cells from a normal, unstimulated syngeneic animal. The results (Table 2) showed no evidence of restoration of the deficiency in column fractionated SL.

TABLE 2

FAILURE TO RESTORE IMMUNODEFICIENCY OF COLUMN-FRACTIONATED SMALL LYMPHOCYTES WITH NORMAL LYMPHOCYTES OR BONE MARROW CELLS IN THE RAT

Donor cells	No. of rats	Geometric mean antibody titre in recipient rats	Range of titres
5×10^6 primed TDL	9	33	<5–480
5×10^6 primed col-SL	11	<5	All <5
5×10^6 primed col-SL + 5×10^6 normal TDL	3	<5	<5–15
5×10^6 primed col-SL + 5×10^6 normal bone marrow cells	3	<5	All <5
5×10^6 normal TDL	6	<5	<5–10
5×10^6 normal bone marrow cells	4	<5	All <5

We have also investigated the capacity of unstimulated TDL and col-SL to mount a primary adoptive immune response. Here our efforts were impeded by the fact that relatively large doses of TDL (20×10^6 or more) were required to obtain consistently positive and reproducible titres in recipients. In the preparation of col-SL, yield losses are severe and $20–40 \times 10^6$ cells is the maximum which it is practicable to use for a single recipient animal. As far as they go, our results show that, as with primed cells, column fractionated small lymphocytes from unimmunized animals are severely immunodeficient. For example, from two separate experiments, eight irradiated recipients received a dose of 20×10^6 normal TDL and 8 days after antigenic stimulation had a mean titre of 160 (range, 15–6000). When seven corresponding recipients received the same number of col-SL their mean titre at 8 days was <5 (range <5–60).

2. *Studies on the age of small lymphocytes emerging from Shortman glass bead columns*

Since a difference in the antigen reactivity of cells before and after glass bead column fractionation was apparent, an experiment was designed to detect any selective advantage of age for cells passing through the column.

Lymphocyte donors (non-stimulated rats) were injected with [^{3}H]thymidine for 1, 2 or 20 days prior to thoracic duct cannulation. The cells from these individuals were then collected and passed through glass bead columns. Autoradiographs of the pre- and post-column cells were prepared and counted for percentage of total labelled cells and of labelled small lymphocytes after 3–7-week exposures.

One animal received 1 μgc/g 24 hours and 30 minutes prior to cannulation. Thoracic duct cells from this rat showed 10/1000 small lymphocytes labelled and 40/1000 medium plus small lymphocytes labelled. Counts of column fractionated thoracic duct cells, on the other hand, showed that only 5/1000 of the small lymphocytes had incorporated label and when medium lymphocytes were added to the count there were still only 8/1000 cells labelled.

When the lymphocyte donor had been given 1 μc/g [^{3}H]thymidine 48 and 24 hours prior to cannulation, 14/1000 of the thoracic duct small lymphocytes were labelled. This proportion was unchanged in the cells emerging from the glass bead columns. Similarly, when the donor had received 1 μc/g [^{3}H]thymidine daily for 20 days before cannulation its lymph showed 194/1000 labelled small lymphocytes and this remained the same after the cells were fractionated. These results confirm the view that the column retains large, medium and perhaps the very youngest of the small lymphocytes (cells <24 hours of age).

3. *Potency of incubated small lymphocytes in adoptive immune responses*

Gowans (1962) described a method of culturing thoracic duct lymphocytes for 24 hours *in vitro* to produce a relative enrichment of the small lymphocyte population. We used this as a second method of partial cell 'purification' in our flagellar antigen system. Cells which had been cultured were compared with the normal population stored over the same period at 4° in irradiated hosts given an antigenic challenge.

Lymph was collected from primed rats and these cells (Fig. 2) when injected at doses of 5×10^6 or above, allowed the recipients to produce high levels of circulating antibody. Storage in medium 199 for 24 hours appeared not to diminish their effectiveness. When these primed lymphocytes underwent agitated culture for 24 hours their viability was reduced to 30–40 per cent viable (by dye exclusion). However, when these incubated-SL were injected (in doses which had been increased to allow for the reduced viability) they produced antibody titres only slightly lower than those of control stored TDL (Fig. 2).

Two tests were made on the stored and shaken cells to confirm that storage had produced a relative enrichment of the population for small lymphocytes. Radioactive marker uptake *in vitro* showed that 24 hours shaken culture reduced the number of cells capable of incorporating [^{3}H]thymidine from 3·6 per cent (in stored cells) to $<0{\cdot}1$ per cent. In fact, no labelled large or medium cells were seen in the autoradiographs of the 24-hour cultured cells, even when slides were scanned for prolonged periods. Tests with [^{3}H]uridine incorporation, however, showed that there was no significant difference between stored and cultured cells in the proportion of viable cells taking up this label.

Once again, only limited observations were made on unstimulated normal TDL compared with normal inc-SL for immune potency. We observed no significant differences in

adoptive responses obtained with equivalent numbers of fresh TDL, inc-SL or of TDL stored for 4, 8, 14 or 24 hours.

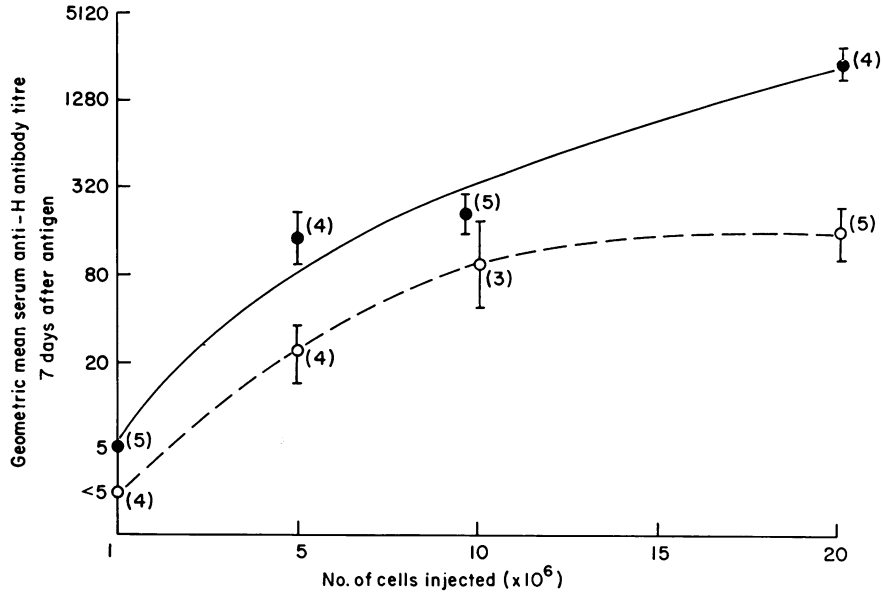

FIG. 2. The effect of agitated culture on primed rat thoracic duct lymphocytes transferred to irradiated syngeneic rats which were challenged 1 day later. ○, Cells which had been in agitated culture for 24 hours prior to injection (inc-SL); ●, untreated TDL stored at 4° for 24 hours. Numbers in parentheses indicate the number of animals at each point. Vertical bars represent standard deviation.

B. MOUSE THORACIC DUCT LYMPHOCYTES

1. *Potency of column fractionated small lymphocytes in adoptive immune responses*

Thoracic duct cells from primed mice conferred on irradiated recipient mice the ability to produce high levels of antibody after challenge when injected in doses over 2×10^6 cells. Table 3 shows that passage of mouse cells through a Shortman glass bead column yielded

TABLE 3

COMPARISON OF UNFRACTIONATED MOUSE THORACIC DUCT LYMPHOCYTES (TDL) AND COLUMN-FRACTIONATED SMALL LYMPHOCYTES (col-SL) IN THE MOUSE

Cells transferred	No. of cells ($\times 10^6$)	TDL		Col-SL	
		No. of animals	Mean serum titre (range)*	No. of animals	Mean serum titre (range)*
Primed	0·5	4	4(<5–16)	4	<5(all<5)
	1	4	8(<5–16)	4	<5(<5–48)
	2	5	130(24–480)	6	32(12–120)
	5	2	320(240–480)	5	35(12–64)
	10	3	400(150–1920)	4	130(40–960)
Normal	0·1	6	<5(all<5)		
	0·5	6	<5(<5–6)		
	1	4	<5(<5–12)		
	2	5	6(<5–16)		
	5	6	9(6–24)	6	<5(all<5)
	10	9	34(12–256)	4	<5(<5–5)
	20	4	313(64–768)	1	6

* Mean serum titre 6 days after antigen in the recipients.

col-SL about five-fold less efficient than TDL in their ability to confer adoptive antibody production.

When thoracic duct lymphocytes were taken from unstimulated mice the results were qualitatively similar; however, a larger number of total cells was required to confer the same level of circulating antibody (Table 3). Due to the technical difficulties of collecting large numbers of mouse thoracic duct lymphocytes higher cell doses were not studied. However, it is clear that the column fractioned small lymphocytes are severely depleted in immune reactivity by a factor of around 20, compared with the control lymphocytes.

2. *Potency of incubation purified small lymphocytes in adoptive immune responses*

Shaken culture of primed mouse cells yielded results shown in Table 4. With primed cells there was a ten-fold reduction in the capacity of cells to confer antigen reactivity after they had been in agitated culture for 24 hours. The overall reduction was of the same order after shaking as after passage through the Shortman glass bead column, although the actual antibody titres were higher due to the later time of bleeding and differences between groups of donors.

TABLE 4

COMPARISON OF PRIMED THORACIC DUCT LYMPHOCYTES STORED AT 4° AND INCUBATED SMALL LYMPHOCYTES IN THE MOUSE

No. of viable cells injected intravenously	Stored TDL		Incubated small lymphocytes	
	No. of animals	Mean serum titre (range)*	No. of animals	Mean serum titre (range)*
1×10^6	4	80(30–480)	5	8(<5–120)
5×10^6	6	2560(640–9600)	3	320(120–600)
10×10^6	6	7000(960–25, 600)	6	700(320–2560)

* Mean serum titre 7 days after antigen.

Limited experimentation has been carried out on the immune reactivity of inc-SL from unprimed mice. It was found that 10×10^6 transferred stored-TDL allowed the emergence of a mean serum antibody titre of 32, whereas 7 days after antigen, inc-SL gave a mean titre of 24, not significantly different. The experiments, though preliminary, indicated that unstimulated inc-SL were *not* as immunodeficient as unstimulated col-SL.

In view of the results of Gallily and Feldman (1967), who have suggested that part of the reason for the immunological defect in X-irradiated animals lies in damaged macrophages, we have performed two small experiments designed to show that the poor reactivity of normal mouse col-SL is not due to an absence of a phagocytic cell type which might be present amongst TDL. In the first experiment, TDL were passed through a Rabinowitz (1964) type of column, which allowed the passage of large and medium lymphocytes but which should have retained whatever monocyte-type cells might have been present. As Table 5 shows, cells emerging from such a column are equally as effective as TDL in mounting a primary adoptive immune response. Such an experiment cannot eliminate the possibility that TDL contain a macrophage precursor which does *not* adhere to glass. The second experiment was an attempt to restore the immunodeficiency of col-SL by an intentional admixture of 2×10^6 mouse peritoneal exudate cells, rich in macrophages. The peritoneal cells by themselves gave a positive adoptive response and failed to augment the response of col-SL.

TABLE 5

ATTEMPTS TO SHOW THAT THE IMMUNODEFICIENCY OF UNSTIMULATED MOUSE COLUMN-FRACTIONATED SMALL LYMPHOCYTES IS NOT DUE TO A LACK OF MACROPHAGES

Cell inoculation	No. of mice	Serum antibody titre 6 days after antigen
10×10^6 normal TDL	9	34 (geometric mean, range 12–256)
10×10^6 column fractionated SL	3	<5, <5, <5
10×10^6 TDL fractionated by Rabinowitz column	3	20, 20, 80
2×10^6 peritoneal macrophages	3	<5, 6, 12
10×10^6 col-SL + 2×10^6 peritoneal macrophages	3	<5, 24, 32

3. *Verification of absence of dividing cells amongst mouse col-SL and inc-SL*

Autoradiographic data showed that mouse cells behaved in the same manner as rat cells in *in vitro* culture with labelled thymidine and uridine. Agitated culture resulted in a virtual abolition of cells capable of incorporating [^{3}H]thymidine whereas over 60 per cent of the cells were still capable of incorporating [^{3}H]uridine. The only difference noted in the mouse lymphocyte cultures was that 24 hours *in vitro* at a cell concentration of $50–100 \times 10^6$/ml reduced the viability to 60–70 per cent of the original, compared with 30–40 per cent viable after culture of rat lymphocytes.

DISCUSSION

While several methods exist for the preparation of populations of lymphocytes free of 'large' and severely depleted in 'medium' sized cells, only two of these methods have been combined with an extensive analysis of the immunological properties of the resulting cell populations. The first method is that of Gowans (1962), which makes use of the fact that the larger, more rapidly metabolizing lymphocytes exhibit a geater sensitivity to adverse tissue culture conditions. Thus, when TDL were taken in high concentration and cultured in a medium with low serum content, it was found that all cells capable of cell division died off rapidly, leaving a population of practically pure small lymphocytes (inc-SL). Gowans has shown that these cells can mount a graft-*versus*-host attack, and reflect the antibody-forming status (tolerant, normal or immune) of the intact donor animal when adoptively transferred. The chief disadvantages of this method are its empiric nature, the presence in the final cell preparation of dead cells and cell debris which militate against detailed cell-size distribution analysis by Coulter counter; and the unknown effects of 12–24 hours of tissue culture under adverse conditions on the residual small lymphocyte population. Yield losses in our hands have varied from 30 to 70 per cent.

Shortman (1966) has devised a different method for preparing pure small lymphocytes which selects cells apparently solely on the basis of their size. It depends on filtering the suspension through a column of fine glass beads under carefully controlled conditions. It has been shown that this method enriches for all cells $< 7{\cdot}5\ \mu$ in diameter, and discriminates against all larger cells. In our hands, the method has yielded populations essentially free of cells incorporating [^{3}H]thymidine (col-SL). The advantages are that the method rests on well-understood biophysical principles; that it yields cells of high viability and functional capacity, free of all cell debris; and, being performed in the cold, it involves no metabolism in the separated cells. The chief (and very considerable) disadvantage of the

method is that it involves a great yield loss—85–90 per cent of all TDL being retained on the column. As well as the practical disadvantage involved in such a loss, there is the theoretical possibility that certain small lymphocytes are 'more sticky' under the filtration conditions than others, i.e. that cells are retained on the basis of some property other than size. Using this method, Shortman and colleagues have annotated the immunological properties of column fractionated small lymphocytes (Shortman and Szenberg, 1969).

The present paper concerns the question of the capacity of the two types of small lymphocyte populations, column-fractionated and incubation selected, to respond to a stimulus with the antigen, polymerized flagellin (POL). A simple adoptive transfer system to syngeneic, lethally irradiated hosts has been used. Controls have shown conclusively: (a) that our hosts were incapable of themselves responding detectably to the antigen, and (b) that TDL from pre-immunized donor animals do not manufacture detectable antibody without further antigenic stimulation. The experiments involved TDL of two species, rat and mouse, and though most effort was concentrated on adoptive *secondary* responses, the capacity of lymphocytes from non-immunized animals also was studied.

In contrast to Gowans and Uhr (1966), who found that inc-SL were actually better than TDL in their capacity to transfer antigen-reactivity to the tail fibre protein of phage ΦX174, we found that incubated small lymphocytes were sometimes equal to and sometimes less effective than TDL in conferring reactivity to POL. In the rat, inc-SL were slightly less effective than TDL in conferring secondary reactivity and not detectably different from TDL for transfer of primary reactivity. In the mouse, the deficiency of inc-SL from pre-immunized animals was considerable, being slightly greater than in rats; and again no significant difference between unstimulated inc-SL and TDL was noted. On the basis of the results overall, one can conclude that in our system the unstimulated antigen-reactive cells survive agitated culture quite well, and a proportion of the 'memory' antigen-reactive cells do also. Thus Gowans' conclusion that some antigen-reactive cells, at least, are 'small lymphocytes' is supported.

TABLE 6

IMMUNOPOTENCY OF SMALL LYMPHOCYTE POPULATIONS: SUMMARY

Species	Antibody response	Type of cell	Immunopotency
Rat	1°	TDL*	++
		col-SL	0
		inc-SL	++
	2°	TDL	++++
		col-SL	±
		inc-SL	+++
Mouse	1°	TDL	++
		col-SL	0
		inc-SL	++
	2°	TDL	++++
		col-SL	++
		inc-SL	++

*For abbreviations see text.

In three of the four test systems the results with column fractionated small lymphocytes were different. Both normal and primed rat col-SL and also normal mouse col-SL were severely immunodeficient; primed mouse col-SL were deficient by a factor about equal to that in primed mouse inc-SL. For convenience, the results are summarized in Table 6.

In view of extensive collateral evidence on the viability ([^{3}H]uridine incorporation and dye exclusion) and functional integrity of col-SL (Shortman, 1968b), we do not feel the poor capacity of col-SL to respond to POL is due to poor overall health. We believe, rather, that the column has discriminated against one or more types of cells important in the transfer of the response. In the memory situation, it is possibly that large rapidly-cycling cells are of some importance as inc-SL also displayed some relative immune deficiency. In the case of primary adoptive responses, this is not a likely explanation, as inc-SL worked quite well in both species. Conceivably a cell type important in the transfer of the response is a non-dividing 'small lymphocyte' of somewhat larger than median size. Alternatively, there may exist one or more subpopulations of 'small lymphocytes' which, for reasons which are still not apparent, may be retained by the glass bead column yet may survive agitated culture. We have sought for evidence that the deficiency in col-SL may be not in antigen-reactive cellls but in some collaborating, non-information-bearing cell type from, for example, bone marrow or peritoneal fluid, but no such evidence was forthcoming.

The strongest statement that can come forward from our results is that pure small lymphocytes from the thoracic duct cannot be regarded as a homogeneous population. Were they so, then inc-SL and col-SL should have behaved identically. The results stress the importance of devising better fractionation techniques for various functionally distinct groups of lymphocytes, and the need for caution in extrapolating from one immunological test system to another.

The best current hope for achieving improved results appears to lie firstly in the use of a technique which would distribute and recover the whole population on the basis of size (Peterson and Evans, 1967); secondly, in the use of a technique which would separate by other criteria, such as density (Shortman, 1968a). The latter has already produced fractionation of functionally different populations of antigen sensitive cells (Haskill, Legge and Shortman, 1969).

ACKNOWLEDGMENTS

This work was supported by Grants from The National Health and Medical Research Council, Canberra, Australia; The National Institutes of Health, AT-O-3958; The United States Atomic Energy Commission, AT(3O-D-3695); and The International Atomic Energy Agency, 470/RB.

REFERENCES

Ada, G. L., Nossal, G. J. V., Pye, J. and Abbot, A. (1964). 'Antigens in Immunity. I. Preparation and properties of flagellar antigens from *Salmonella adelaide*.' *Aust. J. exp. Biol. med. Sci.*, **42**, 267.

Boak, J. L. and Woodruff, M. F. A. (1965). 'A modified technique for collecting mouse thoracic duct lymph.' *Nature (Lond.)*, **205**, 396.

Gallily, R. and Feldman, M. (1967). 'The role of macrophages in the induction of antibody in X-irradiated animals.' *Immunology*, **12**, 197.

Gowans, J. L. (1962). 'The fate of parental strain small lymphocytes in F_1 hybrid rats.' *Ann. N.Y. Acad. Sci.*, **99**, 432.

Gowans, J. L. and McGregor, D. D. (1965). 'The immunological activities of lymphocytes.' *Progr. Allergy*, **9**, 1.

Gowans, J. L. and Uhr, J. W. (1966). 'The carriage of immunological memory by small lymphocytes in the rat.' *J. exp. Med.*, **124**, 1017.

Haskill, J. S., Legge, D. G. and Shortman, K. (1969). 'Density distribution analysis of cells forming 19-S haemolytic antibody in rats.' *J. Immunol.* **102**, 703.

Lind, P. E. (1969). 'Influence of neonatal thymectomy on antibody production in the young adult rat.' *Int. Arch. Allergy*, (In press).

Miller, J. F. A. P. and Mitchell, G. F. (1968). 'Cell to cell interaction in the immune response. I. Hemolysin forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes.' *J. exp. Med.*, **128**, 801.

Mitchell, G. F. and Miller, J. F. A. P. (1968). 'Cell to cell interaction in the immune response. II. The

source of hemolysin forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes.' *J. exp. Med.*, **128**, 821.

MITCHELL, J. (1964). 'Autoradiographic studies of nucleic acid and protein metabolism in lymphoid cells. I. Differences amongst members of the plasma cell sequence.' *Aust. J. exp. Biol. med. Sci.*, **42**, 347.

MITCHELL, J., MCDONALD, W. and NOSSAL, G. J. V. (1963). 'Autoradiographic studies of the immune response. III. Differential lymphopoiesis in various organs.' *Aust. J. exp. Biol., med. Sci.*, **41**, 411.

NOSSAL, G. J. V., CUNNINGHAM, A., MITCHELL, G. F. and MILLER, J. F. A. P. (1968). 'Cell to cell interaction in the immune response. III. Chromosomal marker analysis of single antibody-forming cells in reconstituted irradiated or thymectomized mice.' *J. exp. Med.*, **128**, 839.

NOSSAL, G. J. V. and MÄKELÄ, O. (1962). 'Autoradiographic studies on the immune response. I. The kinetics of plasma cell proliferation.' *J. exp. Med.*, **115**, 209.

NOSSAL, G. J. V., SHORTMAN, K. D., MILLER, J. F. A. P., MITCHELL, G. F. and HASKILL, J. S. (1967). 'The target cell in the induction of immunity and tolerance.' *Cold Spring Harb. Symp. quant. Biol.*, **32**, 369.

PETERSON, E. A. and EVANS, W. H. (1967). 'Separation of bone marrow cells by sedimentation at unit gravity.' *Nature* (*Lond.*), **214**, 824.

RABINOWITZ, Y. (1964). 'Separation of lymphocytes. polymorphonuclear leucocytes and monocytes on glass columns, including tissue culture observations.' *Blood*, **23**, 811.

RUHENSTROTH-BAUER, G. and LÜCKE-HUHLE, C. (1968). 'Two populations of small lymphocytes.' *J. Cell Biol.*, **37**, 196.

SHORTMAN, K. (1966). 'The separation of different cell classes from lymphoid organs. I. The use of glass bead columns to separate small lymphocytes, remove damaged cells and fractionate cell suspensions.' *Aust. J. exp. Biol. med. Sci.*, **44**, 271.

SHORTMAN, K. (1968a). 'The separation of different-cell classes from lymphoid organs. II. The purification and analysis of lymphocyte populations by equilibrium density centrifugation.' *Aust. J. exp. Biol. med. Sci.* **46**, 375.

SHORTMAN, K. (1968b). 'The separation of lymphocyte populations on glass bead columns.' *Symposium on Modern Methods of Macromolecules and Particles.* Madison, Wisconsin.

SHORTMAN, K. and SZENBERG, A. (1969). 'The size and density distribution of fowl blood lymphocytes initiating a graft-versus-host reaction.' *Aust. J. exp. Biol. med. Sci.*, **47**, 1.

WILLIAMS, G. M. (1966). 'Antigen localization in lymphoid states. I. Localization pattern following chronic thoracic duct drainage.' *Immunology*, **11**, 467.