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Fractionation of Immunocompetent Spleen Cells by Albumin Density Gradient Centrifugation

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Summary. Fractionation of spleen cells was performed on albumin density gradients. Larger cells localized in the low density fractions. 19S and 7S antibody producing cells against SRBC and *Esch. coli* endotoxin were found to be enriched in low density fractions; high density fractions being depleted of such cells. Antigen-sensitive cells for humoral antibody synthesis were studied in transfer system and were found to be enriched in the high density fractions. Cells initiating graft-*versus*-host reactions were also enriched in these fractions. The results suggest that the antibody-producing cells are physically distinct from the antigen-sensitive cells initiating humoral antibody synthesis and cell-mediated immunity as well.

INTRODUCTION

It seems established that different cell types participate in various stages of the immune response. Thus, various *in vivo* studies suggested that the cells secreting antibodies are different from those responding to the initial contact with antigen (antigen-sensitive cells) with regard to life span and antigen-dependence (for discussion see Möller, 1969). Cellular interactions of different types have been claimed to be necessary for the induction of the immune response. Of particular interest is the recent demonstration with certain antigens, such as heterologous red cells and serum albumins, that antibody synthesis is facilitated by interaction between bone marrow derived cells and thymus-processed cells, the former differentiating into immunoglobulin synthesizing cells, the latter responding by division in the absence of antibody production (for reviews see Miller and Mitchell, 1969; Davies, 1969; Claman, 1969; Taylor, 1969). Additional evidence for the participation of various cells in the induction of immunity has come from experiments on induction of antibody synthesis in tissue culture, where two or more cell-types are needed for an adequate immune response (Mosier, 1968a, b).

In order to analyse the cellular events in antibody production it is valuable to be able to separate cells according to various physical or biological properties. A major achievement in this direction is represented by the introduction of density gradient techniques for separation of lymphoid cells (Szenberg and Shortman, 1966; Shortman, Hashill, Szenberg and Legge, 1967; Raidt, Mishell and Dutton, 1968). The present study represents an attempt to separate antibody producing and antigen-sensitive cells by the density gradient technique described by Raidt *et al.* (1968). In addition, studies were made on the localization of cells initiating cell-mediated immunity using the graft-*versus*-host assay. The cells in the various fractions were also characterized according to morphology and size.

MATERIALS AND METHODS

Mice

Inbred strains A, ASW, C57BL, C57L, B10·5M, CBA and various F_1 hybrids between these were used.

Immunization

Experiments were performed with sheep red blood cells (SRBC), endotoxin from *Esch. coli* 055:B5 and mouse *H*-2 histocompatibility antigens. Mice were immunized i.v. with 4×10^8 sheep red blood cells suspended in balanced salt solution (BSS). For the endotoxin experiments the animals were immunized i.v. with a bacterial vaccine as described previously (Britton and Möller, 1968). Immunizations against histocompatibility antigens were carried out by the subcutaneous and intraperitoneal inoculation of spleen and lymph node cells from *H*-2 incompatible donors as described previously (Möller and Möller, 1962). At least 3 weekly injections were given prior to the experiments.

Cell suspensions

Prepared from spleens by pressing the organs through a 60-mesh stainless steel screen into BSS. The cells were washed once in BSS and the number of trypan blue unstained cells were counted in a haemocytometer.

Agar plaque assay

The agar plaque assay for the detection of cellular antibody synthesis against SRBC and *Esch. coli* endotoxin was performed as described by Jerne and Nordin (1963) for determination of direct (19S) plaque forming cells (PFC) and as described by Dresser and Wortis (1965) for the detection of indirect (7S) PFC. The developing rabbit anti-mouse γ-globulin serum was prepared by immunizing rabbits with antigen–antibody complexes as described previously (Möller, 1968). For enumeration of PFC against endotoxin, SRBC were coated with endotoxin as described (Möller, 1965) and subsequently used in the assay. Only direct PFC can be detected against this antigen.

Density gradient centrifugation

The technique described by Raidt *et al* (1968) was used. Two different albumin preparations were employed: a 30 per cent solution of bovine serum albumin (Armour Pharmaceutical Co., Chicago, U.S.A.) and human serum albumin (Kabi, Stockholm, Sweden) obtained as a powder. This powder was dissolved to a concentration of 37 per cent W/V in BSS. With both preparations the cell suspension to be analysed was first mixed with 10 per cent albumin and thereafter centrifuged. The supernatant was decanted and the cell pellet, containing 50×10^6 cells, was resuspended in 1 ml of 30 per cent BSA or 37 per cent HSA. Then 1 ml of each albumin concentration (29, 26, 23 and 10 per cent) was added sequentially.

The albumin gradients were made in plastic tubes, fitting a Spinco SW39 rotor. After centrifugation at 20,000 ***g*** for 40 minutes, the interphase between the various concentrations of albumin contained the cellular fractions. One fraction was obtained in the bottom of the tube and four fractions above this. The fractions, numbered from the top, were removed by a syringe and immediately washed once in BSS.

Transfer experiments

Cells obtained from spleens before or after separation by density gradient centrifugation were suspended to 50×10^6 cells/ml and mixed with the antigen (either 0·1 ml concentrated SRBC or 10^9 organisms of a *Esch. coli* vaccine). A volume of 0·25 ml of this suspension was subsequently injected i.v. into irradiated (35–600 r) syngeneic mice. Spleens from the recipients were removed 7 days after transfer and tested for the number of PFC. Occasionally spleen cells from such animals were used for density gradient centrifugation.

Graft-versus-*host experiments*

Twenty to 40×10^6 cells derived from various fractions after centrifugation were inoculated into newborn F_1 hybrids or allogeneic mice. Each recipient was given 1 or 2×10^6 cells in a volume of 0·05 ml. Allogeneic recipients were maximally 2 days old, whereas semi-syngeneic F_1 hybrids were up to 7 days old. Each litter was divided in three groups: one received cells from fraction 5, one cells pooled from fractions 1, 2 and 3 and one served as an untreated control group. Seven days after injection, the animals were weighed and their spleen and livers taken out and weighed separately. A splenomegaly (hepatomegaly) index (Simonsen and Jensen, 1959) was calculated as follows: the weight (mg) of the spleen (liver) per gram body-weight was first determined (relative organ weight). Subsequently the relative spleen and liver weights of the various groups were used to determine the graft-*versus*-host index according to the formula: relative organ weight in the experimental group, divided by the relative weight in the control group. An index of 1 indicates that the control and experiemental group had the same relative organ weight, whereas values above one indicate an enlargement of the organs in the experimental group.

Histology

Cells to be studied were suspended to 10^6 cells/ml and 0·1 was added to a cyto-centrifuge and centrifuged into microscope slides at a speed of 500 rev/min. They were subsequently air dried, fixed in methanol and stained with haematoxylin and eosin.

Cell size

The spleen cell suspensions were diluted to a final volume of 4·0 ml. A volume of 0·5 ml of a 0·1 per cent solution of saponine in BSS was added to the cells for 3 minutes. Thereafter an equal volume of BSS was added to the suspension which was subsequently centrifuged at 1500 rev/min for 12 minutes. In order to remove cell-debris the cells were resuspended in a 0·5 per cent solution of trypsin in BSS and allowed to stand for 60 minutes at 37°. After centrifugation at 1500 rev/min for 12 minutes and resuspension in BSS the cells were counted and their size-distribution determined. This was performed in a Coulter Counter to which was connected an electronic particle size-distribution plotter. The latter makes a graphic representation of the proportion of cells within each size category. The height of all peaks on the graph was added and the percentage of each peak of the total was calculated.

RESULTS

CELL SIZE IN POPULATIONS ENRICHED FOR ANTIBODY-PRODUCING CELLS

As described previously (Möller, 1968) a large increase of the proportion of antibody-producing cells can be obtained by transfer of hyperimmune spleen cells mixed with SRBC

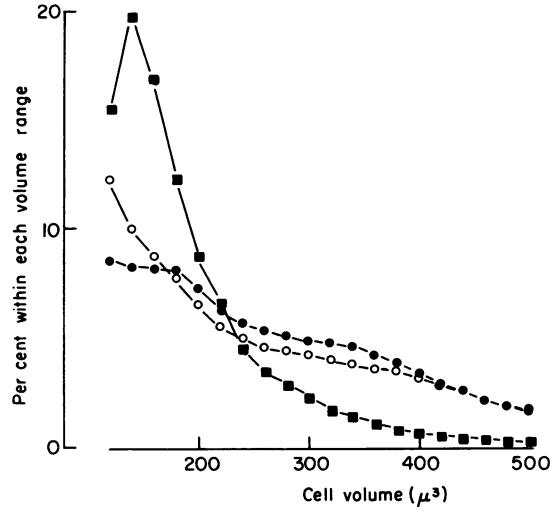

FIG. 1. Size-distribution diagram of spleen cells from normal mice (■) and from X-irradiated mice given syngeneic spleen cells alone (○) or with SRBC (●) 7 days earlier. Each point represents the mean value of three different experiments with fifteen animals.

into irradiated recipients. Seven days later the spleen of such animals contain between 1 and 10 per cent antibody-producing cells as a rule. The size of the cells in such spleens, as compared to control animals given spleen cells without antigen, was analysed by an electronic particle size distribution platter. In three different experiments with fifteen animals it was found (Fig. 1) that there was an increase in the proportion of large cells in the antigen-stimulated spleen cells, containing a high percentage (1·60±0·55 per cent) of antibody-producing cells, as compared to the proportion of large cells in non-antigen

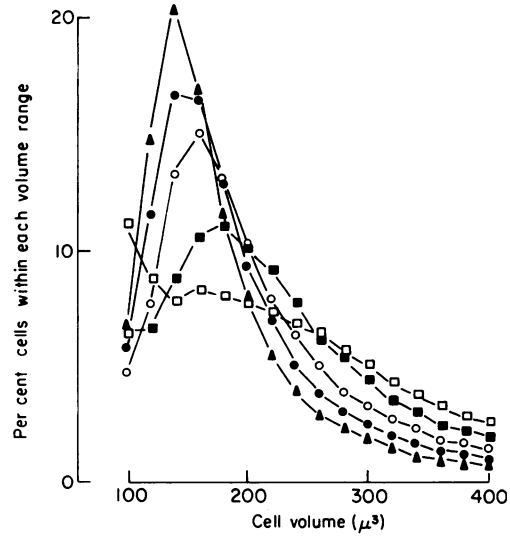

FIG. 2. Coulter counter analysis of different fractions after separation of spleen cells on albumin density gradients. The following symbols are used: Fraction 1 (□) 2 (■) 3 (○) 4 (●) and 5 (▲). Each point represents the mean value of six to seven experiments.

treated spleen cells having a much lower fraction of antibody producing cells (0·0028+ 0·001 per cent). A higher proportion (62·6 per cent) of the cells in the antigen-treated groups were larger than 180 μ^3 as compared to 56·5 per cent in the control groups. Thus, 6·1 per cent of the cells had increased in size as a consequence of antigen-stimulation. The proportion of large cells was nearly four times greater than the fraction of antibody producing cells.

TABLE 1

MORPHOLOGY OF CELLS IN VARIOUS FRACTIONS AFTER DENSITY GRADIENT CENTRIFUGATION

Fraction No.	Distribution (per cent) between fractions of		Distribution (per cent) within fractions of mononuclear cells			
	Red cells	Granulocytes	Small lymphocytes	Medium-large lymphocytes	Blast cells	Plasma cells
1	0	0	3–8	47–56	37–39	4–6
2	0	5–40	16–20	26–55	4–20	0, 1–5
3	0	10–15	25	58	17	0
4	10	35–75	33–56	44–55	0–11	0
5	90	10	90–95	5–10	0	0

At least 300 mononuclear cells were counted on each slide in two different experiments and classified as small lymphocytes, medium to large lymphocytes, blast cells and plasma cells. The distribution of red cells and granulocytes between the different fractions was subjectively estimated.

This finding suggested that separation of spleen cells on density gradients may be of value in order to enrich antibody producing cells even further. As a first step, spleen cells from immunized or normal animals were added to albumin density gradients and the size distribution of the various fractions was studied. It was found that fraction 5 was markedly depleted of large cells, the dominating size present being that of a small lymphocyte. The proportion of larger cells increased towards the top fractions, although these also contained smaller cells (Fig. 2).

The morphology of the cells in the various fractions was also studied. The bottom fraction (5) contained red cells, dead cells and small lymphocytes. The number of larger lymphocytes, lymphoblasts and plasma cells increased towards the top of the gradient. Granulocytes were found mostly in fractions 2, 3 and 4 (Table 1).

DISTRIBUTION OF ANTIBODY-PRODUCING CELLS

More than fifty gradient separations were carried out and, therefore, only representative experiments will be documented below. Direct and indirect PFC were studied in the various fractions after separation of spleen cells from mice immunized against endotoxin and SRBC. As can be seen from Table 2, the highest proportion of antibody-producing cells were found in fractions 1 and 2, whereas few or none were found in fraction 5. Although there was considerable variation between different experiments, most of the cells usually localized in fractions 3 and 4, whereas 1 and 5 contained a smaller number. These findings are in general agreement with those of Raidt *et al.* (1968).

Enrichment, defined as the proportion of antibody-producing cells in a particular fraction, divided by the proportion in the original suspension, was found to be largest in fractions 1, 2 and 3. Two experiments are illustrated in Fig. 3. It was regularly found that fraction 5 was virtually depleted of antibody producing cells. As can be seen in Table 2

TABLE 2

PLAQUE-FORMING CELLS AFTER SEPARATION OF SPLEEN CELLS ON ALBUMIN GRADIENTS

Strain	Immunized against	State of immunization	Gradient	Fraction No.	Per cent cells* per fraction	19S PFC		7S PFC	
						Per 10^6 cells	Per cent per fraction*	Per 10^6 cells	Per cent per fraction
A × CBA	*Esch. coli*	Transfer†	BSA	2	8·7	5139	81·2		
				3	9·1	974	16·2		
				4	16·8	49	1·4		
				5	65·4	7	1·1		
CBA	SRBC	Transfer	BSA	2	3·8			24·668	54·0
				3	16·5			4·157	39·8
				4	11·8			393	2·7
				5	67·9			90	3·6
CBA	SRBC	Primary‡	HSA	1	2·4	5421	4·2		
				2	22·6	6349	45·2		
				3	15·4	7000	34·1		
				4	37·0	1250	14·6		
				5	22·6	230	1·6		
CBA	SRBC	Hyperimmune§	HSA	1	18·8			123	24·7
				2	29·1			205	63·4
				3	33·8			30	10·6
				4	15·0			8	1·3
				5	3·2			0	0
CBA	SRBC	Hyperimmune	HSA	1	0·9			333	13·3
				2	3·3			76	11·1
				3	12·8			35	20·0
				3	43·1			29	55·6
				5	39·9			0	0
A	SRBC	Hyperimmune	HSA	1	18·5			70	2·0
				2	20·1			1589	48·6
				3	28·7			1113	48·6
				4	22·9			184	6·4
				5	9·8			51	0·8
A × CBA	SRBC	Hyperimmune	HSA	1	5·1	1·2	1·0	20·3	1·1
				2	44·1	11·3	86·5	185·5	86·4
				3	26·6	2·3	10·8	33·5	9·4
				4	17·1	0·4	1·3	17·4	3·1
				5	7·1	0·3	0·4	0	0

* Number of cells (PFC) in a particular fraction × 100 divided by the total number of cells (PFC) in all fractions.
† Hyperimmune spleen cells (10^7) were mixed with the corresponding antigen *in vitro* and thereafter inoculated i.v. into irradiated (350 r) syngeneic recipients. The recipient spleens were used for the experiments 7 days later.
‡ Spleens taken 5 days after a primary immunization.
§ Spleens taken from animals given at least 3 weekly injections with SRBC, the last given 7 days prior to the experiment.

there was no difference between the localization of direct and indirect PFC. Similar results were obtained with PFC against SRBC and endotoxin. When spleen cells from irradiated animals given antigen-stimulated immune cells were fractionated a very high proportion of PFC was recorded in the two top fractions.

DISTRIBUTION OF ANTIGEN-SENSITIVE CELLS

A transfer system was used to study the presence of antigen-sensitive cells. Cells in the various fractions were mixed with the antigen and transferred into irradiated syngeneic recipients. As shown previously (Möller, 1968), few or no PFC can be detected in the secondary host unless the cells are stimulated with antigen, indicating that the cells actually producing antibody are short-lived. In general, it was found (Table 3) that

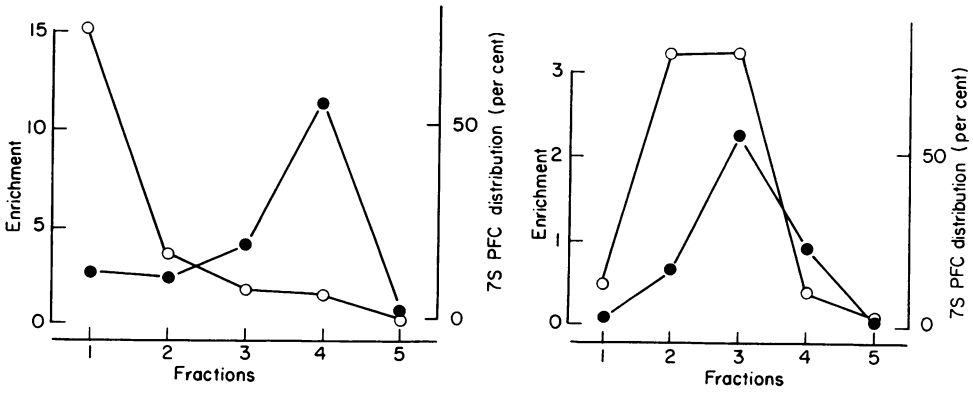

FIG. 3. 7S PFC against SRBC in various fractions after separation of immune spleen cells on human serum albumin gradients. Enrichment (○) is defined as No. of PFC/10^6 cells in a fraction divided by the No. of PFC/10^6 cells in the unfractionated population. ●, Indicates the proportion of PFC in a fraction as related to the total number of PFC recovered. (No. of PFC per fraction divided by the total number of PFC in all fractions.)

TABLE 3

7S PFC IN IRRADIATED RECIPIENTS INOCULATED 7 DAYS EARLIER WITH FRACTIONATED SPLEEN CELLS MIXED WITH SRBC

Donor strain	Immune status of donor	Fraction No.	7S PFC/10^6 inoculated cells ($\log_{10}$)	7S PFC/10^6 in recipient ±SE ($\log_{10}$)	No of PFC produced after transfer per PFC inoculated	Relative No. of PFC†
CBA	Normal	1	–	1·8066±0·15		1
		2	–	2·2175±0·10		3
		3	–	2·5908±0·16		6
		4	–	3·5651±0·09		57
		5	–	4·3897±0·01		383
5M	Normal	1	–	1·0450±0·15		1
		2	–	0·8864±0·17		0·7
		3	–	1·2602±0·20		2
		4	–	1·3694±0·21		2
		5	–	3·0370±0·03		98
CBA	Immune‡	1	2·0899	3·1260±0·06	11	1
		2	2·3118	3·4800±0·06	15	3
		3	1·4771	3·8292±0·05	225	6
		4	0·9031	3·6720±0·29	588	4
		5	0·0000	4·0880±0·22	12300	9
A×5M	Immune	1	1·3222	2·1858±0·13	7	1
		2	2·1492	3·4951±0·09	22	20
		3	2·1818	3·8163±0·11	43	43
		4	1·9445	4·0733±0·21	134	77
		5	1·3222	3·0680±0·22	56	8
CBA	Transfer§	2	4·3927	2·1965±0·19	0·02	1
		3	3·6191	3·1758±0·05	0·28	10
		4	2·5944	2·9630±0·01	1·9	5
		5	1·9542	3·2692±0·02	20·7	12

* The number of 7S PFC produced in the recipients was divided by the number of 7S PFC inoculated.
† The number of 7S PFC in each group was divided by that found in the recipients of cells from fraction 1.
‡ Hyperimmune animals given at least 3-weekly injections with SRBC.
§ Spleen cells from irradiated (350 r) mice, which 7 days earlier were inoculated with SRBC mixed with syngeneic spleen cells from hyperimmune donors.

fractions 4 and 5 gave rise to a large number of antibody-producing cells, whereas fractions 1 and 2 were depleted of cells capable of initiating an immune response (Fig. 4). This was true whether the cells were derived from non-sensitized or immunized animals. However, the immune status of the donor played a role in the distribution of antigen-sensitive cells. As can be seen from Table 3, the relatively largest concentration of antigen-sensitive cells in non-sensitized spleen cells was found in fraction 5, the other fractions containing much less. However, after immunization the relative concentration of antigen-sensitive cells was more evenly distributed between the fractions, suggesting that these cells differentiated as a consequence of antigen confrontation. This finding is in agreement with those of Raidt *et al.* (1968) obtained in tissue culture systems.

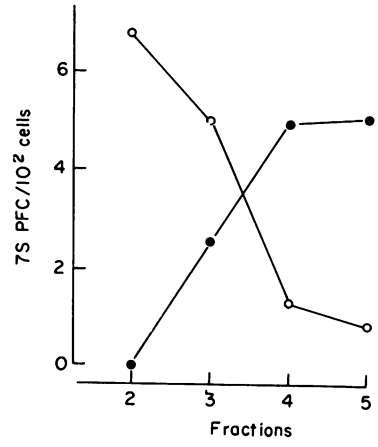

FIG. 4. 7S PFC/10^2 cells against SRBC in various fractions after density gradient centrifugation on HSA gradients of spleen cells derived from irradiated recipients given immune spleen cells mixed with SRBC 7 days earlier (○) and 7S PFC/10^2 cells in irradiated recipients given cells from the various fractions mixed with SRBC 7 days earlier (●).

Similar experiments were also performed with spleen cells obtained after transfer of antigen-stimulated immune cells into irradiated recipients. Such cells are markedly enriched in antibody-producing cells. These populations were relatively depleted of antigen-sensitive cells (Table 3). Thus, a lower number of antibody-producing cells was obtained after transfer as compared to the result with immune cells. This suggests that the marked increase in antibody-producing cells obtained after the first transfer may have decreased the pool of antigen-sensitive cells.

GRAFT-*versus*-HOST REACTIVITY

To study the localization of cells capable of mediating cell-mediated immunity, experiments were performed with the graft-*versus*-host assay as described by Simonsen and Jensen (1959). The assay system did not allow the study of all fractions simultaneously. Therefore, fractions 1, 2 and 3 were combined and compared with fraction 5. The results can be seen in Table 4. The cells mediating the graft-*versus*-host reaction were found in fraction 5 to a larger extent than in fractions 1, 2 and 3.

TABLE 4

GRAFT-*versus*-HOST ACTIVITY OF FRACTIONATED SPLEEN CELLS IN NEWBORN RECIPIENTS

Strain		Fraction inoculated	Graft-*versus*-host index* ±SE	
Donor	Recipient†		Spleen	Liver
CBA anti-SRBC	A	2–3	1·09	1·07
	A	5	1·78±0·13	1·99±0·18
CBA anti-SRBC	A	2–3	0·96	1·11
5M anti-A	A	1, 2, 3	1·29±0·06	1·28±0·03
	A	5	1·40±0·01	1·56±0·07
5M anti-A	A	1, 2, 3	1·01±0·05	1·13±0·07
	A	5	1·69±0·07	1·86±0·09
ASW anti-A	A×C57L	1, 2, 3	1·18	1·92
	A×C57L	5	1·74±0·04	3·39±0·26
ASW anti-A	A×C57L	1, 2, 3	1·85±0·28	1·06±0·08
	A×C57L	5	2·41±0·20	1·21±0·10
ASW anti-A	A×C57BL	1, 2, 3	0·98	1·35
	A×C57BL	5	1·60±0·11	1·67±0·08
5M anti-A	A×5M	1, 2, 3	1·24±0·04	1·21±0·15
	A×5M	5	1·46±0·06	1·44±0·09
Total		1, 2, 3	1·20±0·08 (20)‡	1·25±0·06 (20)
		5	1·67+0·07 (23)	1·83+0·14 (24)

* Index = weight of spleen or liver in mg/g body weight in experimental group divided by organ weight in mg/g body weight in uninoculated control. If only two mice were used per group no standard error was calculated.

† Allogeneic recipients were 0–3 days old, whereas semi-syngeneic F_1 hybrid recipients were 0–7 days old.

‡ No. of mice tested within parenthesis.

DISCUSSION

These experiments demonstrate that antibody-producing cells are enriched in the low density fractions and depleted from the high density fraction after albumin density gradient centrifugation. This is in general agreement with the findings of Raidt *et al.* (1968). In addition it was demonstrated that larger cells went to the top fractions and small lymphocytes remained in the bottom. The antigen-sensitive cells were enriched in the bottom fraction and less common in the top fractions. Since morphological observations indicated that fraction 5 contained mostly small lymphocytes, whereas various larger cells, including plasma cells and transformed lymphocytes, went up to fractions with lower density, the antigen-sensitive cells may be small lymphocytes, at least in populations from non-immunized mice. As a consequence of immunization, some antigen-sensitive cells appeared to change their density and were found to a greater extent in fractions of lower densities, indicating that these cells also differentiate after antigen stimulation.

The cells inducing the graft-versus-host reaction were also enriched in the bottom fractions. This is in agreement with the findings of Gowans and McGregor (1965), who demonstrated that small lymphocytes initiate graft-*versus*-host reactions and antibody production to SRBC and OX phage. Szenberg and Shortman (1966) using a continuous BSA gradient also concluded that small lymphocytes constituted the major part of the cells inducing the graft-*versus*-host reaction.

The depletion of antibody-producing cells in fraction 5 and the ability of this fraction to give rise to antibody-producing cells after injection into irradiated recipients clearly demonstrate that this fraction contains cells which by themselves, or in collaboration with cells in the recipient, are capable of initiating an adequate immune response. The failure

of the top fractions to achieve this, suggests that these fractions are depleted of antigen-sensitive cells. This cannot be critically proved, however, since it could be argued that they do not lack the initiator cells, but some helper cells, which would be lacking also in the irradiated host.

The results substantiate earlier conclusions (Möller, 1968, Britton and Möller, 1968) that antibody-producing cells represent differentiated cell types, which can divide a few times even in the absence of antigen, but thereafter disappear. It follows from this that the maintenance of the immune response depends on continuous stimulation of new antigen-sensitive cells. Therefore, continuation of antibody synthesis appears to require the continuous presence of antigen.

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