CELLULAR EVENTS IN THE IMMUNE RESPONSE

Analysis and In Vitro Response of Mouse Spleen Cell Populations Separated by Differential Flotation in Albumin Gradients*, ‡

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It has been demonstrated that dissociated spleen cells from normal mice can be immunized in vitro against heterologous erythrocytes (1). The number of antibody-producing cells increases roughly a 1000-fold over the first 4 days in culture as measured by the Jerne hemolytic plaque assay. We have begun to examine cell types, cell interactions, and cell changes involved in the immune response using a cell separation technique in combination with the culture system.

This paper presents a procedure for separation of mouse spleen cells into populations with differing immunologic properties utilizing differential flotation in discontinuous albumin density gradients. A similar method has been reported by Shortman and his colleagues (2–4), the principal differences being that with the technique described here, the pH was maintained at 7.0 instead of 5.1 and the density changes were stepped rather than continuous. Spleen cell populations from both normal and immunized mice were separated into four subpopulations or bands which formed at the interfaces between albumin layers during centrifugation. These bands were assayed for antibody-forming cells by the hemolytic plaque assay and for precursor cells as measured by the size of the response to antigen in the in vitro culture system.

The principal findings were as follows. The majority of antibody-producing cells and precursors taken from unstimulated animals were initially found in denser regions of the gradients. After in vivo stimulation with antigen, both

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antibody-producing cells and precursor cells were found in less dense regions of the gradient.

The cell density change, although gradual, occurs principally in the first 12–18 hr after antigenic stimulation. In an experiment using two non–cross-reacting antigens it was demonstrated that the cells which undergo density change are antigen specific and can be separated by the technique described from resting cells of other antigen specificity. A brief account of these findings has been previously reported (5).

The significance of these results will be discussed.

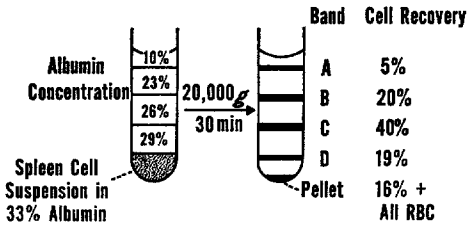

FIG. 1. Schematic representation of cell separation method. Spleen cell suspensions were incorporated into 33% albumin solution and loaded into the bottom of centrifuge tubes. This mixture was overlayered with 29, 26, and 23% albumin solutions and a final layer of 10% albumin was layered on top. Centrifugation was carried out for 30 min at g_{max} = 20,000. Cells which distributed into discrete bands on the basis of differential flotation were recovered with a Pasteur pipet. Approximate cell recovery was as indicated.

Materials and Methods

Mice.—2- to 3-month-old mice, BDF_1 hybrid (C57BL/6 female × DBA/2 male) taken from our own colony were used in all experiments. Stocks were obtained from Jackson Laboratories, Bar Harbor, Me.

Immunizations.—Mice were immunized by intravenous injection of 2×10^8 erythrocytes.

Reagents.—Sterile bovine serum albumin was obtained from two sources. Three lots of 35% BSA without sodium azide, catalog BV0862, were obtained from Pentex Corporation, Kankakee, Ill. Sterile 30% BSA was kindly donated by Reheis Chemical Co. (Chicago, Ill.). Dilutions of albumin stocks were prepared on a volumetric basis using balanced salt solution as diluent. Balanced salt solution contained dextrose, 1000 mg; KH_2PO_4, 60 mg; $Na_2HPO_4 \cdot 7\ H_2O$, 358 mg; phenol red, 10 mg; $CaCl_2 \cdot 2\ H_2O$, 186 mg; KCl, 400 mg; NaCl, 8000 mg; $MgCl_2 \cdot 6\ H_2O$, 200 mg; $MgSO_4 \cdot 7\ H_2O$, 200 mg to 1000 ml in distilled water. Culture reagents and antigens were as previously described (1).

Cell Separation Method.—An outline of the cell separation method is shown in Fig. 1. Spleens were removed from mice killed by cervical dislocation and teased in balanced salt solution using sterile technique. Fragments of spleen and cell aggregates were allowed to settle out and remaining cells were centrifuged in conical centrifuge tubes at 4°C and 1000 rpm for 10 min. One part packed cells was resuspended in nine parts of 33% albumin and the mixture was loaded in the bottom of a Spinco centrifuge tube. In a few early experiments cells were loaded in the top layer. If small numbers of cells were needed, 1.0 ml volumes containing approximately 2×10^8 cells were loaded into each of three 5-ml cellulose nitrate tubes for use in the Beckman Spinco SW 39 rotor. The cell mixture was then carefully overlayered

with 1.0 ml quantities of 29, 26, and 23% albumin with a final spacing layer of 0.5 ml of 10% albumin. If larger cell recoveries are required, 7.0 ml of cell mixture (approximately 1.4×10^9 cells) could be loaded in 35-ml cellulose nitrate tubes for use in the Beckman Spinco 25.1 rotor. In this case, 5.0-ml volumes of the less dense albumins were used for overlayering.

TABLE I

Total Cell Recovery from Bands A, B, C, and D as Per Cent of Number Loaded

Cell loading	Albumin	Recovery	Mean recovery
		%	%
Top	Lots 39, 40 (Pentex)	89.8, 102.4, 82.2, 98.0,	89.2
	Lot A60807 (Reheis)	73.8	
Bottom	Lots 39, 40 (Pentex)	83.6, 83.3 81.5, 80.4	84.0
	Lot A60807 (Reheis)	92.2, 86.3 82.4, 82.4 83.7	
Bottom	Lot 41 (Pentex)	66.4, 56.5 65.9, 61.5	62.6

TABLE II

Cell Distribution Expressed as Per Cent of Number Loaded

Albumin	Recovery*				
	A	B	C	D	Pellet‡
	%	%	%	%	%
Lots 39, 40 (Pentex)	9.3	23.4	39.1	10.5	17.7
	3.0	17.6	50.8	11.0	17.6
	6.7	27.8	40.6	11.2	13.7
Lot A60807 (Reheis)	5.9	24.2	37.7	24.2	8.0
	2.6	10.7	40.4	26.8	19.5
	2.3	18.2	33.7	27.4	18.4
	4.1	20.7	40.3	18.1	16.8
Mean.....	4.8	20.4	40.4	18.5	16.0
Lot 41 (Pentex)	1.1	4.5	15.3	44.9	34.2
	3.5	9.0	23.0	30.9	33.6
Mean.....	2.3	6.8	19.2	37.9	33.9

* Data from normal and primed mice are pooled. No significant difference could be observed.

‡ Calculated by difference (total loaded minus sum of cells recovered from bands A, B, C, and D).

Centrifugation was carried out at 4°C for 30 min at an rpm which produced a g_{max} = 20,000 (tip of the tube). Discrete bands of cells which formed at the interfaces between albumin solutions of different densities were easily harvested with a sterile Pasteur pipet. "A" band cells (or the "A" subpopulation) were defined as the population of cells which were harvested from the interface above the 23% albumin layer; "B" band cells were harvested from the interface above 26% albumin; "C" band cells were harvested from the interface above 29% albumin; and "D" band cells were harvested from the interface above 33% albumin. Other cells sedimented to the bottom of the tube and were recovered as a pellet. Cells were then washed two times in a large excess of balanced salt solution before assay or culture.

TABLE III

Recovery of Plaque-Forming Cells after Separation of Spleen Cells from In Vivo Primed Mice

Exp.	Day immunized prior to sacrifice	No. of PFC loaded	PFC recovery	Recovery
				%
36	3	2.16×10^4	2.80×10^4	130
38	3	3.94×10^4	3.66×10^4	93
39	3	5.18×10^4	5.19×10^4	100
Mean				108
40	2	3.7×10^3	2.6×10^3	70
43	2	16.8×10^3	15.5×10^3	92
Mean				81
42	1	1.0×10^3	1.3×10^3	130
Mean of all data				103

Assay.—Cell counts were made by hemocytometer or Coulter Counter. Antibody or plaque-forming cells (PFC) were measured by the hemolytic plaque technique of Jerne (6) as modified by Mishell and Dutton (1). The bands were assayed immediately to measure the number of antibody-forming cells. The culture response as determined by assay for plaque-forming cells after 3 or 5 days of culture with antigen was taken as a measure of the number of precursor cells which had been present initially, with the reservations discussed below.

Culture Method.—$1.2–1.8 \times 10^7$ cells from the whole spleen cell population (SC) or separated populations (A, B, C, or D) were cultured with 3×10^6 sheep erythrocytes by the method of Mishell and Dutton (1). In certain cases, one part separated cells were mixed with three parts normal cells to give a total density of $1.2–1.8 \times 10^7$ cells per ml of culture.

RESULTS

Cell Recovery.—Although cell recovery was somewhat variable, more than 80% of all spleen white cells loaded into the gradient were recovered among the bands (Tables I and II). The pellet contained all the red cells and the remainder (10–16%) of the white cells. The latter contained only occasional

plaque-forming cells and did not survive well in culture. There was some variation between lots of albumin and one lot, 41 (Pentex), gave lower total cell recovery in the four bands and more cells in the pellet; however, the biological properties of the cell populations produced in the separation procedure were not significantly different. It can be seen that almost half of the cells were

TABLE IV

Relative Distribution of Plaque-Forming Cells

The figures indicate PFC per 10^6.

Source of spleen cells	Exp.	Whole spleen cell suspension	Subpopulations			
			A	B	C	D
Spleen cells from unimmunized mice	37	1.2	6.2	2.9	0.6	0.5
	52	6.2	15.0	8.5	2.7	1.4
	63	3.0	14.5	10.2	8.3	1.1
Spleen cells from mice immunized 1 day prior to separation	35	4	58	13	2	7
	42	2	32	1.1	0.5	0.7
	51	3	24	5	2.4	0
Spleen cells from mice immunized 2 days prior to separation	34	75	594	62	15	17
	40	7	83	16	1	5
	43	33	351	22	2	1
	49	11	67	11	1.3	0.7
Spleen cells from mice immunized 3 days prior to separation	36	40	619	102	10	9
	38	115	784	159	37	31
	39	119	1465	238	39	29
	48	61	240	84	32	3

recovered in band C. Bands B and D contained approximately one-fifth each while relatively few cells were found in band A.

Antibody-Forming Cells.—Virtually all plaque-forming cells loaded into the gradient were recovered among the bands after separation of spleen cell suspensions prepared from in vivo primed mice (Table III). The distribution of plaque-forming cells among bands as PFC/10^6 is given in Table IV. The per cent distribution of total plaque-forming cells is given in Table V. The average enrichment of plaque-forming cells, and average total distribution of plaque-forming cells after separation of normal and primed mouse spleen cells are illustrated in Figs. 2 and 3. Although there is some enrichment in background plaque-forming cells in the A band after separation of normal spleen cells (Table IV and Fig. 2), most plaque-forming cells are found in the much larger B and C bands (Table V and Fig. 2). After stimulation with antigen by in-

jection 1, 2, or 3 days prior to sacrifice, however, the A population is more strikingly enriched for plaque formers (Table IV and Fig. 3) and contains roughly half of the plaque-forming cells from the starting spleen cell population (Table V and Fig. 3). The C and D bands were depleted of plaque-forming

TABLE V

Total Distribution of Plaque-Forming Cells

The figures indicate the total number of PFC in each band expressed as a per cent of the PFC recovered.

Source of spleen cells	Exp.	Subpopulations			
		A	B	C	D
Spleen cells from unimmunized mice	52	21.6	31.4	33.2	13.8
	63	6.8	19.3	53.1	20.7
Mean		14.2	25.4	43.1	17.3
Spleen cells from mice immunized 1 day prior to separation	35	27.4	34.6	16.3	21.6
	42	74.8	10.5	7.7	7.1
	51	60.3	22.6	17.1	0.0
Mean		54.2	22.6	13.7	9.6
Spleen cells from mice immunized 2 days prior to separation	34	25.7	63.4	9.0	1.9
	40	41.8	39.8	7.9	10.5
	43	77.0	20.0	2.7	0.4
Mean		48.2	41.1	6.5	4.3
Spleen cells from mice immunized 3 days prior to separation	36	48.7	40.5	7.7	3.1
	38	50.1	39.8	8.1	2.0
	39	33.9	44.5	13.5	8.2
	48	49.2	14.0	35.7	1.1
Mean		45.5	34.7	16.3	3.6

cells. The A populations produced from stimulated mice contain 10-fold more plaque-forming cells than the unseparated spleen cells, while in general the C and D bands contain 10-fold fewer antibody-producing cells than the starting population.

Precursor Cells.—The populations described above were then cultured with antigen and assayed at 3 or 5 days. The assumption was made that the best culture response would be found in that population of cells which contained the greatest number of specific precursor cells.

In order to interpret the results of culture experiments using separated cells,

it was necessary to know whether the albumin was toxic to the cells, or would interfere with the expected culture response. For this purpose, cells from normal and primed animals were prepared as usual. Half of each pool was incorporated into 33% albumin, held at 4°C for 2 hr, and then washed twice with

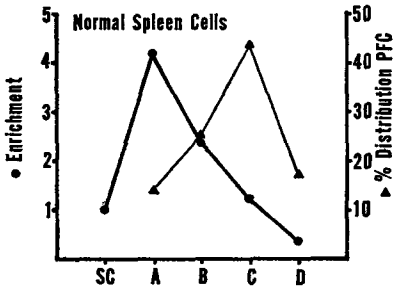

FIG. 2. Distribution of plaque-forming cells after separation of spleen cells from unimmunized mice.

Enrichment, ●—●; i.e., the number of PFC per 10^6 cells in each band (A, B, C, and D) relative to the number of PFC per 10^6 cells in the unseparated spleen cell population, SC.

Per cent distribution PFC, ▲—▲; i.e., the number of PFC in each band expressed as per cent of the total PFC from all the bands.

Values represent the average of three experiments.

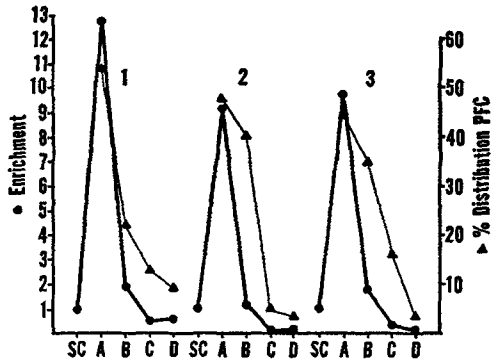

FIG. 3. Distribution of plaque-forming cells after separation of spleen cells from mice injected with sheep erythrocytes 1, 2, or 3 days prior to sacrifice.

For symbols, see legend to Fig. 2.

excess balanced salt solution; this corresponded to the treatment received by gradient-separated cells. The remaining half were held at 4°C without treatment. Previous studies (unpublished) had shown that holding cells at 4°C for several hours had little effect on the culture response. The response of cultures of these cells is shown in Fig. 4. As can be seen, there was no significant dif-

ference between the response of albumin-treated and untreated cells. Furthermore, if the band cells were remixed and then cultured, the response of band cells was always slightly better than untreated controls.

Cells from different bands showed no significant differences in capacity to survive in culture based on cell recovery after 3 or 5 days of culture (Table VI). If the total culture response of band cells is compared with the response of equivalent numbers of unseparated cells, it can be seen that the development of plaque-forming cells is not significantly different between these two groups

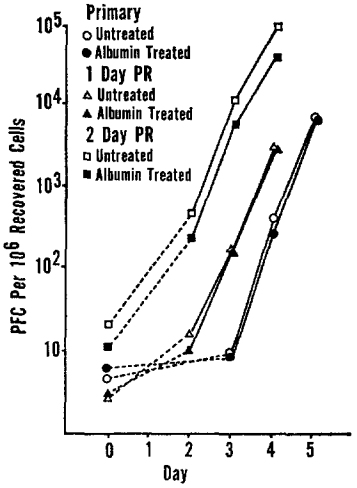

FIG. 4. Culture response of control and albumin-treated spleen cell populations obtained from unimmunized mice or mice primed (PR) 1 or 2 days prior to sacrifice. For details of the experimental procedure see text.

(Table VII). The results of culture experiments are given in Table VIII. There was considerable variation in the response of any given pool of spleen cells; however, the patterns of the response were consistent within each experimental design. The A band cells of cultures from unimmunized mice always gave poorer responses than the whole spleen cell suspension or C or D population. The A subpopulation separated from immunized mice gave stronger responses than the starting population, while C and D subpopulations gave markedly diminished responses. The average enrichment in antigen-sensitive cells among bands as measured by the development of PFC in culture, and the average per cent total distribution of antigen-sensitive cells are given in Figs. 6 and 7.

Frequently, the A band cells represented less than 5% of cells recovered from the gradient. This meant that the number of A cultures which could be prepared was severely limited. For this reason we investigated the possibilities

of mixing A band cells from gradient separations of primed cells with normal cells. A cells derived from the separation of spleen cells obtained from mice immunized 3 days prior to sacrifice were mixed 1 + 99, 1 + 19, and 1 + 3 with normal cells and cultured with antigen in the usual manner. The kinetics of the

TABLE VI

Cell Recovery From Bands After Culture as Per Cent of Cells Planted

Exp.	Day immunized prior to sacrifice	A	B	C	D	SC	Culture period
							days
37	Unimmunized	18	38	26	35	35	5
44	Unimmunized	51	40	50	40	40	5
52	Unimmunized	45	50	43	35	35	5
63	Unimmunized	20	20	28	35	41	5
Mean		34	37	37	36	38	
35	1	45	52	42	25	52	3
42	1	48	40	66	45	36	3
Mean		47	46	54	35	44	
34	2	66	52	54	41	73	3
40	2	37	52	43	30	35	3
43	2	88	80	69	57	53	3
Mean		64	61	55	43	54	
36	3	42	62	40	39	44	3
39	3	48	46	69	43	62	3
Mean		45	54	55	41	53	
Mean of all Exps		53	55	55	40	51	

culture response to sheep erythrocytes of these mixtures is shown in Fig. 5. If the PFC/10^6 are expressed per A cells added to the culture, it can be seen that the response was quantitatively the same at the cell densities used. Furthermore, in a number of experiments, A, B, C, and D band cells were cultured either directly or mixed 1 + 3 with normal spleen cells for comparison. In all cases, the response in PFC/10^6 of the mixed cultures was about 25% of the response of the corresponding band cultured directly. In later experiments with primed animals, therefore, A, B, C, or D band cells were mixed with normal cells, and the response was adjusted to 100% by multiplying by the appropriate factor.

TABLE VII

Comparison of the Culture Response of Separated Cells with the Culture Response of the Original Cell Suspension

Day immunized prior to sacrifice	Exp.	Band	Total PFC developed in individual bands	Response of equivalent No. of unseparated spleen cells	Response
					%
1	35	A	64,999		
		B	10,710		
		C	31,160		
		D	7,519		
Sum			114,388	112,225	102
1	42	A	484,920		
		B	323,244		
		C	28,650		
		D	11,439		
Sum			848,253	545,923	155
1	51	A	68,472		
		B	52,136		
		C	41,328		
		D	21,504		
Sum			183,440	333,984	55
2	40	A	312,884		
		B	668,628		
		C	168,504		
		D	1,080		
Sum			1,152,096	5,207,356	22.1
2	43	A	1,393,456		
		B	668,058		
		C	34,608		
		D	11,514		
Sum			2,107,763	2,859,264	74
3	36	A	940,900		
		B	2,436,670		
		C	441,070		
		D	11,250		
Sum			3,829,890	3,736,590	102
3	39	A	156,312		
		B	1,161,284		
		C	277,200		
		D	72,708		
Sum			1,667,500	1,548,600	108
3	48	A	1,003,200		
		B	563,620		
		C	1,157,500		
		D	181,880		
Sum			2,906,240	4,699,040	63

Since the change in density of cells responding to antigen occurs within the period 24 hr after stimulation, further experiments were made to define more closely the period in which the cell density changed. Mice were sacrificed and cells separated at 6-hr intervals after in vivo stimulation and cultured with antigen. The results of these experiments are shown in Fig. 8. There is a gradual shift in cell density until 18 hr when 40% of precursor cells are found in the A band.

TABLE VIII

The Figures Represent PFC/10^6 after Culture of Subpopulations and Unseparated Mouse Spleen Cells Obtained From Normal Mice or Mice Immunized at Various Times Prior to Sacrifice

	Exp.	Whole spleen cell suspension	Subpopulations			
			A	B	C	D
5-day culture responses of	37	909	148	207	1,154	377
spleen cells from unimmu-	44	4,662	776	2,652	4,454	5,958
nized mice	52	6,888	168	1,096	1,325	7,175
3-day culture responses of	35	335	5,909	170	164	103
spleen cells from mice im-	42	1,169	16,164	2,628	150	93
munized 1 day prior to	51	392	1,268	532	164	48
separation						
3-day culture responses of	34	3,848	17,301	111	1,200	416
spleen cells from mice im-	40	12,826	24,068	12,382	826	8
munized 2 days prior to	43	6,528	40,984	4,738	168	202
separation	49	1,299	22,971	864	360	269
3-day culture responses of	36	8,378	42,768	21,952	2,042	116
spleen cells from mice im-	38	10,500	85,000	8,000	885	350
munized 3 days prior to	39	3,560	13,026	11,972	1,540	498
separation	48	10,928	20,900	14,452	4,035	2,218

Response to Two Non-Cross-Reacting Antigens.—We examined the specificity of the immune response of the different subpopulations in an experiment using two non-cross-reacting antigens. Mice were injected with sheep erythrocytes and separated by the gradient method 1 day later. Cultures of cells from the different bands were then prepared and stimulated with both sheep and burro red cell antigens. Burro and sheep erythrocytes have been shown to have less than 1% cross-reactivity. Cultures were harvested on day 3 and assayed by the plaque technique against both sheep and burro. As seen in Fig. 9, the same pool of cells gave a "primed" type of response assayed against sheep (largest response in A band) and a primary culture response assayed against

burro (response in D band). The responding cells for the two antigens were thus found to be distributed independently of one another.

Preliminary size distribution analyses on subpopulations of spleen cells have shown the following: the A population contains mostly small cells; the

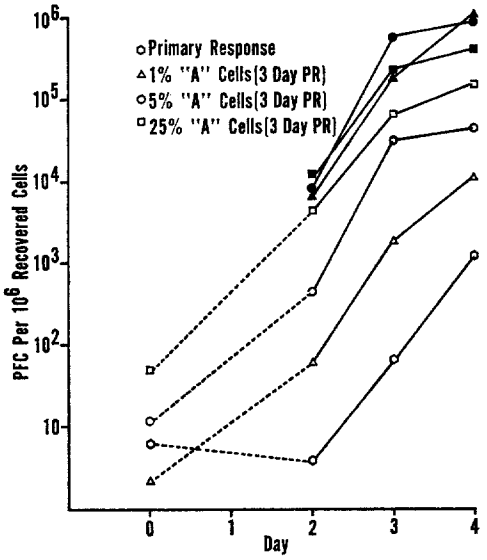

FIG. 5. Culture response of A cells separated from spleen cells obtained from mice injected 3 days prior (PR) to sacrifice with sheep erythrocytes. A cells are mixed as 1% (△—△); 5% (○—○); or 25% (□—□) of the total with normal spleen cells. Solid figures show the results when response is adjusted to 100%.

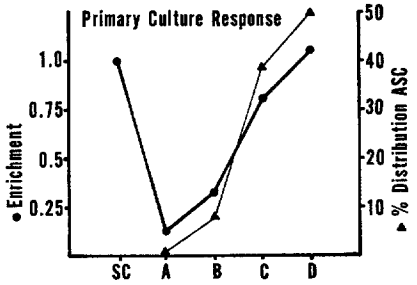

FIG. 6. Culture response of separated cells obtained from the spleens of unimmunized mice. The numbers of PFC were determined on cells harvested after 5 days in culture.

Enrichment, ●—●; i.e., the number of PFC/10^6 in culture bands A, B, C, and D relative to the number of PFC/10^6 in cultures of unseparated spleen cells, SC.

Per cent distribution ASC, ▲—▲; i.e., the total number of PFC in each band expressed as a per cent of the total PFC from all the bands.

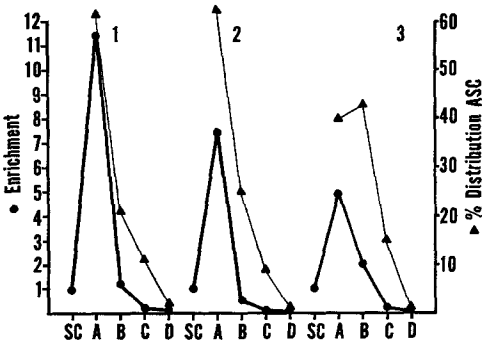

FIG. 7. Culture response of separated cells obtained from the spleens of mice immunized 1, 2, or 3 days prior to sacrifice. The numbers of PFC were determined on cells harvested after 3 days in culture.

For symbols see legend to Fig. 6.

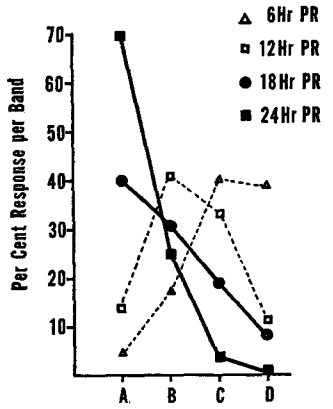

FIG. 8. Culture response of separated spleen cells obtained from mice primed by injection of sheep erythrocytes at various times before sacrifice. The results of four individual experiments are given as the per cent of the total culture response per band.

B population contains small cells and some larger cells; the C population resembles the unseparated spleen cell population; and the D population contains more large cells than the starting population. More extensive studies of size distribution and morphology are in progress and will be reported later.

DISCUSSION

Numerous publications have recently appeared describing the separation of blood leukocytes or cells from lymphoid organs by a variety of techniques. Separations are achieved based on differences in cell sedimentation rate or buoyant density in albumin (2–4, 7–12) or ficoll (13) solutions, cell attachment

to glass (14, 15), nylon (16), plastic (17), cotton (18), or collagen fibers (19), size filtration in glass bead columns (20), and differential electrophoresis (21), and a variety of procedures to remove cells active in phagocytosis (22, 23). A number of sophisticated devices are in the process of development which sort individual cells on the basis of some measurable characteristic such as cell diameter, light scattering, conductivity or absorption at various wavelengths (24, 25). These latter are restricted in use at present since they can handle only very small quantities of cell suspension.

The principle aim of cell separation procedures is to produce cell populations with different biological functions. Very recently, Haskill (26) and Wigzell

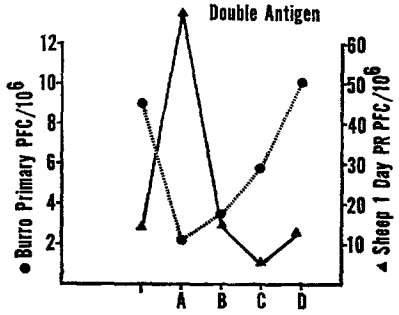

FIG. 9. Culture response of separated spleen cells obtained from mice injected 1 day prior to sacrifice with sheep erythrocytes which were challenged in culture to both the priming antigen and a non-cross-reacting antigen (burro erythrocytes).

(18) have reported separation of plaque-forming and antigen-sensitive cells, and Mosier (17) has shown that spleen cell suspensions can be separated into two populations, each inactive alone, but which can respond if the proper population is exposed to antigen and subsequently recombined with the other.

The procedure that we have developed is similar to that described by Shortman et al. (4). There are, however, some significant differences, both in technique and result. In the Shortman technique the albumin is exhaustively purified and freed of salt. The resulting albumin is dissolved in distilled water and restored to isotonicity with sodium chloride but the pH, 5.1, remains close to the isoelectric point. No effect of this pH on the white cell population has been shown but the erythrocytes present swell and are distributed in the gradient instead of sedimenting in the pellet. In the present study the albumin used is also isotonic but is buffered to pH 7. The erythrocytes are sedimented to the bottom of the gradient and from a comparison of our data and those of Shortman (4) and of Haskill (26) the distribution of the white cell population (as judged by biological function) may also be different. We have used discontinuous steps of albumin concentration instead of a continuous gradient. This results in an obvious loss of potential for resolution but greatly simplifies

the recovery of cells which distribute in discrete bands at the interfaces. For these reasons and the fact that there is a species difference, rat versus mouse, some caution should be exercised in comparisons as to where cells sediment in the two systems.

The data presented clearly demonstrate that the separation method yields a useful enrichment and depletion of both 19S plaque-forming cells and their precursors; i.e., those cells or groups of cells capable of responding to antigen in culture. Both plaque-forming cells and their precursors sediment in the denser regions of the gradient when cell suspensions are made from the spleens of unimmunized mice. There is, however, some enrichment of the background plaque-forming cells in the A band which lends support to the concept that the background plaque formers represent a separate population of cells from the antigen-sensitive precursor cells. After antigenic stimulation both plaque formers and precursors are found predominantly in the A band. This change in density takes place during the first 18 hr after antigenic stimulation and is detectable by 12 hr. It is perhaps the earliest detectable event in the immune response. It is not known what relationship there is, if any, between this density change and the blast transformation of small lymphocytes. This observation thus confirms and is essentially similar to that reported by Haskill (26) using the technique of Shortman.

The most significant finding of this paper lies in the fact that the cell that undergoes a change in density is specific for the antigen bringing about that change and that cells able to respond to other antigens remain in the denser bands of the gradient.

The simplest interpretation of these observations is that an antigen-sensitive, antigen-specific precursor cell changes in density; other interpretations, however, are possible. It is perhaps worth spending some time in discussing those questions that are left unanswered.

Two and probably three components would seem to be necessary for an immune response. These are: (*a*) The precursor cell, identifiable as the cell that gives rise to progeny which engage in antibody synthesis. (*b*) The genetic information specifying the amino acid sequence of the specific antibody ultimately synthesized by the progeny of the precursor cell. (*c*) Another cell type involved in some cooperative activity with the precursor cell.

In the simplest explanation above we have assumed that the second component, i.e. the genetic information specifying amino acid sequence, is already present in the precursor cell so both specificity and precursor cell move together. Several hypotheses, however, have been put forward in which the information is initially present in another cell and is only transferred to the precursor cell after antigen stimulation. Adler, Fishman, and Dray (27) have suggested a transfer of information by RNA from macrophages, Mitchell and Miller (28) a transfer from thymus derived cells, and Smithies (29) has pro-

posed an antibody virus transfer system; none of these hypotheses are excluded by the present data or the earlier demonstration that separate populations are involved in the response to different antigens (30). The suggestion has also been made that a second cell such as the macrophage is involved in an antigen-handling process conveying the antigen to the precursor cell. The data presented here throw no light on this aspect of the problem.

More recent studies have shown that a minimum of two cell types are involved and that these are partially separated by the gradient technique. These will be described in a subsequent publication.[1]

SUMMARY

Cell suspensions from the spleens of normal mice or mice injected with sheep erythrocytes were separated on a discontinous bovine serum albumin density gradient. Four bands or subpopulations were obtained and were assayed for antibody-forming cells, and for antigen-sensitive precursor cells. The antibody-forming cells were assayed by the hemolytic plaque assay and the antigen-sensitive precursors were assayed by the number of plaque-forming cells which developed after 3 or 5 day's culture with antigen. It was found that both antibody-forming cells and their precursors were present in the denser region of the gradient when spleen cell suspensions were taken from unimmunized mice. In contrast, both antibody-forming cells and precursors floated to the top in cell suspensions from mice sacrificed 1, 2, or 3 days after antigen injection. The change in density was detectable as early as 12 hr and was complete by 18 hr. The cell which changed in density was specific for the antigen that brought about that change. The significance of these findings is discussed.

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