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Specific antibody-forming B-lymphocyte colonies

I. DISTRIBUTION AND NATURE OF SRBC ANTIBODY-FORMING B-LYMPHOCYTE COLONIES IN MOUSE LYMPHOMYELOID ORGANS

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Summary. When normal mouse spleen or lymph node cells are cultured for 7 days in agar-medium containing 2-mercaptoethanol and sheep red blood cells (SRBC), approximately one B lymphocyte colony (BLC) develops per 50–100 cells seeded. Incubation of cultures for 3 h with guinea-pig complement at day 7, demonstrated that 0·05–0·25% of all BLC form specific antibody against SRBC (SRBC-AF-BLC). The SRBC specific colonies appear centrally in lytic plaques of 2–5 mm in diameter and cells recovered from individual SRBC-AF-BLC were shown to produce antibodies of the IgM class against SRBC. Cells forming SRBC-AF-BLC are absent in the new-born and infantile spleen but appear in adult mice with a frequency of 5, 10–25 and 25–70 per 10^6 bone marrow, spleen and lymph node cells respectively. Specific immunization *in vivo* or *in vitro* does not greatly affect the number of SRBC-AF-BLC-forming cells. Cytolysis of spleen cells with anti-Ig serum plus complement prior to culture reduced the number of total BLC and that of specific SRBC-AF-BLC by 93% and 94% respectively. The peak sedimentation velocity of both SRBC-AF-BLC-forming cells and total BLC-forming cells was 3·5 mm/h. Spleen cells enriched 200–300 times for cells that bind specifically to the hapten NIP were not enriched for cells forming colonies with specific antibody production against NIP. The data indicate that the cells that give rise to specific antibody-forming colonies belong to a mature virgin B cell group of small Ig-positive B lymphocytes.

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INTRODUCTION

A cloning system for mouse B-lymphocytes in semisolid agar-medium is now well established (Metcalf, Nossal, Warner, Miller, Mandel, Layton & Gutman, 1975). This assay has been used to study various aspects of colony-formation by B-lymphocytes. The nature and foetal development of B-lymphocyte colony-forming cells (BL-CFC) have been elucidated (Metcalf, Wilson, Shortman, Miller & Stocker, 1976; Johnson, Metcalf & Wilson, 1976), and anti-immunoglobulin mediated control of B-lymphocyte colony-formation (Kincade & Ralph, 1976) as well as the age-related frequency of certain B-lymphocyte subsets (Claësson & Metcalf, 1977) have been studied. The results from these studies seem to indicate that the BL-CFC represent

broad range of B-lymphocyte subpopulations from virgin cells of the pre-progenitor or direct-progenitor B cell type (Howard, Claësson & Johnson, 1977) to relatively mature lymphocytes of the memory type. B-lymphocyte colony cells have been shown to secrete immunoglobulins of μ- and light-chain types (Metcalf *et al.*, 1975) indicating that at least some (a few) colonies reach a stage where plasma cell differentiation occurs. The obvious value of this B-lymphocyte cloning system is its inherent potential for enrichment and purification of a given, deliberately chosen functionally characterized B lymphocyte subset.

Consequently, we have used the B-lymphocyte colony system to study that minority of BL-CFC which spontaneous—or as a result of specific stimulation—develop into SRBC-antibody-forming colonies (SRBC-AF-BLC). Individual colony cells recovered from specific SRBC antibody-forming colonies were shown to produce specific lytic plaques when examined with SRBC in the Cunningham–Szenberg plaque-forming cell assay (Cunningham & Szenberg, 1968).

MATERIALS AND METHODS

Mice

Highly inbred CBA/CaH/Wehi mice were used in most experiments. Other strains studied were: AKR, ASW, BALB/c, C_{57}Bl/6, SJL/J, NZB, and nu/nu mice of BALB/c origin. In addition germ free CBA mice were studied.

Immunization

Mice were injected intravenously with 0·2 ml of a 10% SRBC supension in physiological saline.

Agar culture

Cell suspensions were prepared from spleen, lymph nodes and bone marrow using standard techniques by teasing the organs with fine needles in Eisen's balanced salt solution. The required number of eosin-excluding cells was added in aliquots of 0·2 ml to a mixture of double strength Dulbecco/s modified Eagle's medium (DME) with an equal volume of 0·6% Difco Bacto agar. The composition of the medium and conditions of culture have been described previously (Metcalf *et al.*, 1975). Briefly, 1 ml volumes of the cell suspension in agar-medium containing a final concentration of 5×10^{-5} M 2-mercaptoethanol (2-ME) and 0·05 ml of 60% washed SRBC were pipetted into 35 mm plastic dishes, allowed to gel, and incubated for 7 days at 37° in a fully humidified atmosphere of 10% CO_2 in air. On day 7 of culture, 0·5 ml of a 33% solution of fresh guinea-pig serum previously absorbed with SRBC was added to each culture dish and incubation continued for 3 h.

Scoring of cultures

Colonies that lysed SRBC in the agar-medium could be scored with the naked eye as the diameter of individual lytic plaques was 2–5 mm. The size of the individual colonies was studied using an Olympus dissection microscope. Colonies were removed from the centre of the lytic plaques using a fine Pasteur pipette and each transferred to 100 μl Eisen's balanced salt solution. The colony cells were dispersed with a 28 G needle mounted on a 1 ml tuberculin syringe. The single colony cell suspension was tested for direct (IgM) and indirect (IgG) plaque-forming cells (PFC) according to the method of Cunningham & Szenberg (1968). Total number of B-lymphocyte colonies was counted after addition of 3% acetic acid to lyse the red cells.

Liquid cultures

Five million spleen cells/ml were cultured in a pH controlled, humidified incubator, with or without 5×10^7 SRBC, in DME containing 10% FCS and with or without 5×10^{-5} M 2-ME. The cells were cultured in gas permeable poly-fluorethylene membrane bags* and culture volume was 2 ml. The period of culture was 1–4 days. At termination of incubation, the cells were recovered, counted for viability and assayed for SRBC-PFC, and cultured for B-lymphocyte colonies.

Hapten-specific cell fractionation

The method of Haas & Layton (1975) as modified by Nossal & Pike (1976), was used to separate NIP (4-hydroxy-3-iodo-5-nitrophenyl acetic acid)-specific spleen cells. The method enriched specific NIP-binding cells by a factor of 200–300. NIP-enriched, NIP-depleted and unfractionated spleen cells were cultured for specific antibody producing colonies with normal SRBC or NIP-SRBC. NIP-SRBC were made by incubating washed SRBC with NIP-

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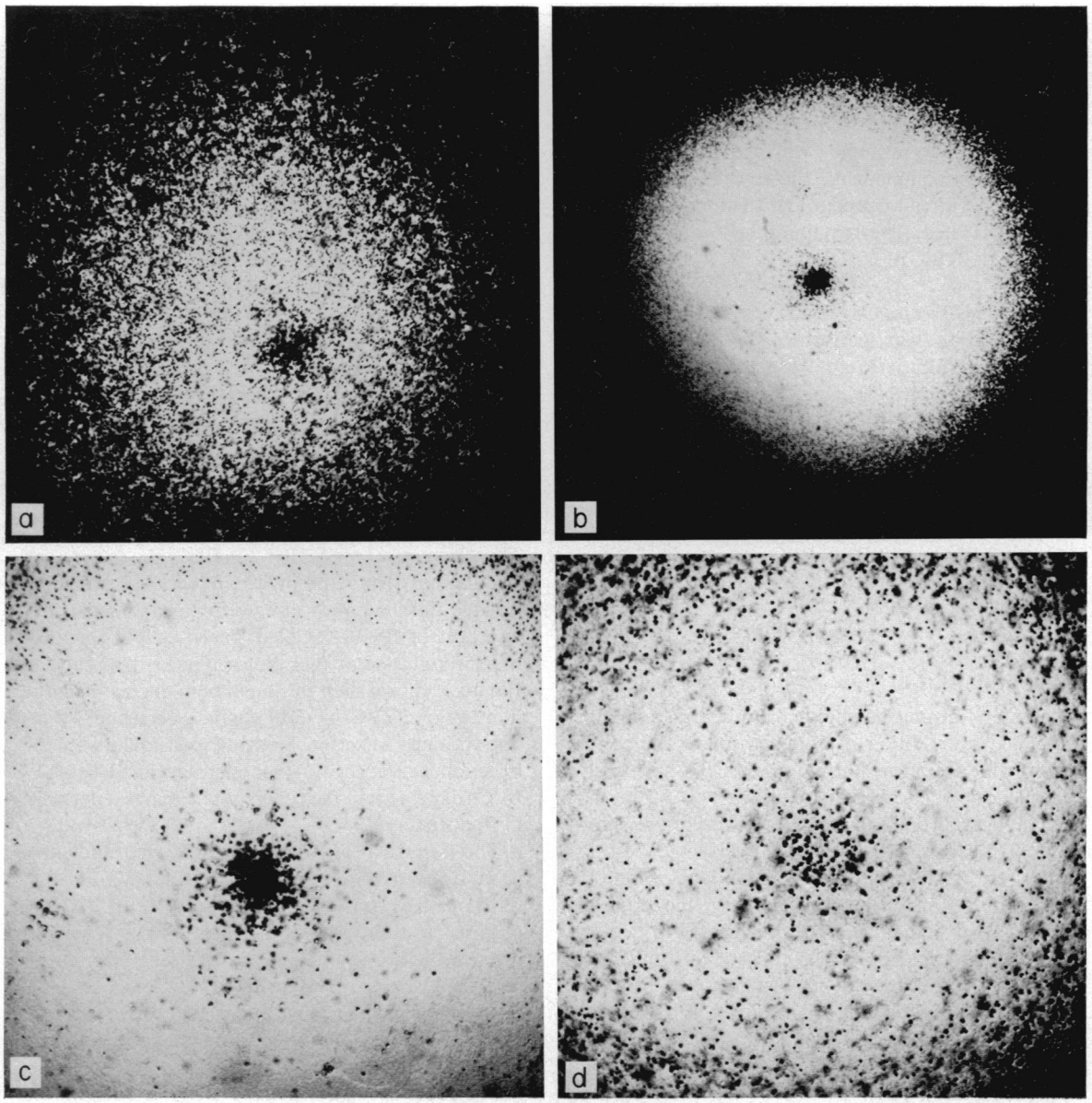

Figure 1. a–d. SRBC-AF-BLC producing varying degrees of lysis of SRBC in agar-medium (Magnification: (a) ×30; (b) ×20; (c) and (d) ×50).

conjugated F(ab)'' fragments of an IgG fraction of rabbit anti-SRBC serum.

Antibody treatment of spleen cells

Five to 10×10^6 spleen cells were incubated at 4° with 10% sheep anti mouse Ig (μ + light chain) serum* for 45 min, then washed and reincubated at 38° with 10% rabbit serum (complement)† for 45 min. Control cells were treated with normal sheep serum followed by complement. Viable cells were counted before and after treatment and the number of killed cells calculated. The remaining cells were then cultured for SRBC-BLC.

Velocity sedimentation

Fractionation was performed by the method of Miller & Phillips (1969), with some modifications as outlined elsewhere (Johnson *et al.*, 1976). Approximately 10^8 spleen cells were added to a 7–30% buffered step gradient of FCS, in an 11 cm glass sedimentation chamber and allowed to sediment at unit gravity and 4° for 3 h. Fractions of 10 ml were collected and the cells washed, counted and plated for total BLC and SRBC-BLC.

RESULTS

SRBC-AF-BLC

Colonies forming haemolytic antibodies to SRBC, detected by addition of complement to the culture dish, were found in normal adult CBA spleen at a frequency of 10–25 per 10^6 cells or 1–3 per 1500 BLC. Typical antibody-forming colonies are shown in Fig. 1. Sometimes only a cell cluster (5–50 cells) was seen in the central part of the lytic plaque. In general, the greater the degree of lysis, the larger the colony and the higher the percentage of colony cells which could be shown to produce anti-SRBC antibody when the colony was removed from the dish and the individual cells assayed in a Cunningham chamber. Figure 2 summarizes the results from an analysis of 30 SRBC-AF-BLC for individual plaque-forming cells. Twelve of the colonies were negative whereas 1–100% of the cells from eighteen colonies produced plaques. Some colonies (8) were divided in two and assayed for both direct and indirect plaques. IgG production was also tested for by adding enhancing serum directly to the colony dish after it had been incubated with complement, to see whether more SRBC-AF-BLC appeared. In neither case was any convincing evidence of IgG production obtained. Control colonies for the Cunningham assay were recovered from the eccentric part of the lytic plaque close to the antibody-forming colony. All were negative.

The incidence of SRBC-AF-BLC in lymphomyeloid organs of mice of different ages was surveyed (Table 1). No antibody-forming colonies were seen in neonatal and infantile mice. The lymph nodes of 5-month-old animals showed the highest frequency with 68 SRBC-AF-BLC per 10^6 lymph node cells or one SRBC-AF-BLC out of 400 BLC. In 1-year-old mice the number of specific colonies seemed to decrease. Germ-free mice had a higher frequency of SRBC-AF-BLC in the spleen than normal mice of the same age, but the frequency in lymph node was lower.

SRBC immunization *in vivo* and *in vitro*

The frequency of SRBC-AF-BLC in spleen cell populations from immunized mice was studied at various time intervals after primary and secondary injection of SRBC. Pooled spleen cells from three mice were tested at each time point in BLC culture. Figure 3 shows that the maximum increase in the number of SRBC-AF-BLC, which occurred 3 days after primary injection, was only two-fold. A second injection caused no further increase in SRBC-AF-BLC frequency; in fact, there was a marked decrease in frequency.

The frequency of SRBC-AF-BLC was studied in seven different mouse strains (AKR, ASW, BALB/c, C_{57}B1/6, SJL/J, NZB, and nu/nu mice of BALC/c origin). In each case, pools of 3 spleens from unprimed mice and mice 3 days after injection of SRBC, were tested. The frequency of specific colonies in the unprimed mice was consistently 3–8 per 10^6 spleen cells and no effect of immunization could be demonstrated in any of these strains.

The effect of *in vitro* culture of spleen cells on the frequency of SRBC-AF-BLC and total BLC is shown in Table 2. Cells were cultured with or without SRBC and with or without 2-ME for 1–4 days. Recovered cells were then assayed for plaque-forming cells (Cunningham assay) and cultured for SRBC-AF-BLC. Neither the presence of SRBC or 2-ME in the pre-culture period influenced the frequency of

* Kindly supplied by Dr N. L. Warner.

† Kindly supplied by Dr J. W. Goding.

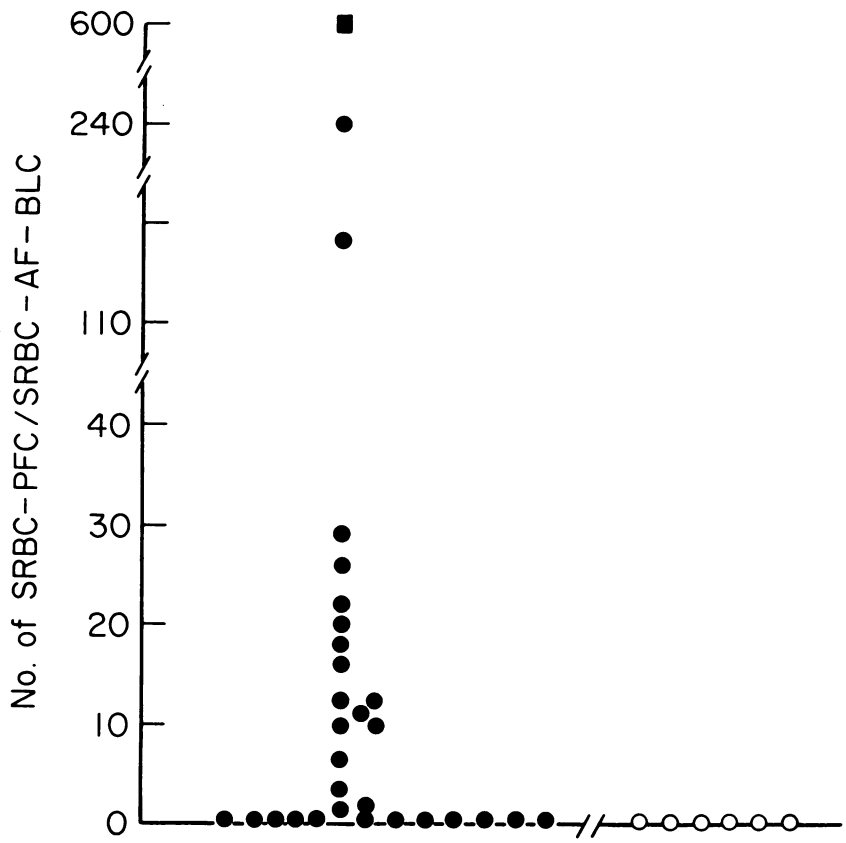

Figure 2. Number of SRBC-plaque-forming cells (PFC) recovered from individual SRBC-AF-BLC (●). The number of PFC from a pool of four specific colonies is included (■). Open circles indicate negative control-colonies. Number of cells per colony = 50 – 200.

specific colonies to any major extent. In contrast, the frequency of SRBC-PFC increased by a factor of 8 in the presence of both 2-ME and SRBC (individual PFC-data not included in Table 2).

Selective killing of Ig-bearing cells

Spleen cells from unprimed CBA mice were treated with an anti-Ig serum plus complement prior to culture in agar for BLC. It has previously been shown that this method does not kill thymus cells (Howard *et al.*, 1977). Table 3 shows the results of a typical experiment. Approximately 50% of the spleen cells were killed, but the treatment reduced the number of total BLC and the number of SRBC-AF-BLC by 93 and 94% respectively, indicating that the majority of colony-forming cells irrespective of their functional potential have Ig receptors on the surface.

Velocity sedimentation

Cell suspensions from spleens of unprimed CBA mice were fractionated by velocity sedimentation and the fractions assayed for ability to form SRBC-AF-BLC. Figure 4 shows the pooled results of two separate experiments. Cells forming SRBC-AF-BLC segregated into a major peak at 3·5 mm/h, whereas peak sedimentation value for nucleated spleen cells was 3·1 mm/h. These figures indicate that most SRBC-AF-BLC-forming cells are small lymphocytes. The sedimentation profile of total BLC coincided with that of the total nucleated cell profile.

NIP-AF-BLC

Spleen cells from unprimed CBA mice can be fractionated on NIP-gelatin coated dishes and the cells which bind are enriched by a factor of 200–300 for

Table 1. SRBC-AF-BLC and total BLC from spleen, mesenteric lymph nodes, and bone marrow of pools of 3–5 CBA mice of different ages. Each data point represents SRBC-AF-BLC and total BLC frequencies counted in 20 and 4 replicate cultures respectively with 25,000 cells per dish. ±SD values are included. The different age-groups were assayed at least twice with essentially similar results obtained

	Colonies per 10^6 cells					
	Spleen		Lymph nodes		Bone marrow	
Mouse age days	SRBC-AF-BLC	Total§ BLC	SRBC-AF-BLC	Total BLC	SRBC-AF-BLC	Total BLC
0*	0	4·700 ± 650	n.t.		n.t.‡	
7	0	8·350 ± 1050	n.t.		n.t.	
15	4 ± 2	8·600 ± 850	n.t.		n.t.	
60	10 ± 3	14·600 ± 280	24 ± 5	22·930 ± 3·490	5 ± 2	3·850 ± 210
60†	40 ± 16	24·700 ± 3600	7 ± 3	8·500 ± 1·180	0	5·730 ± 950
150	26 ± 5	20·650 ± 1400	68 ± 12	28·900 ± 2·720	5 ± 3	3·700 ± 400
360	3 ± 2	26·650 ± 2300	6 ± 2	24·550 ± 1·850	n.t.	

* Pools of 3–4 mice.
† Germ free mice.
‡ n.t.: not tested.
§ Mean value of four replicate cultures.

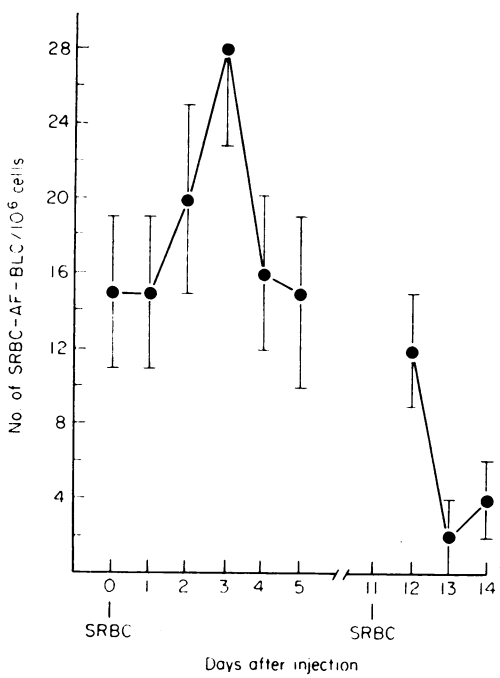

Figure 3. Effect of one and two intravenous injections of 0·2 ml 10% SRBC on the frequency of spleen SRBC-AF-BLC. Each data point represents a pool of three spleens. Specific colonies were counted in twenty replicate cultures containing 25,000 cells per dish. Bars represent ±SD.

specific NIP-binding cells (Haas & Layton, 1975). At least 20% of BLC from NIP-enriched cells has been shown to bind NIP-polymerized flagellin (Metcalf *et al.*, 1975). NIP-enriched, NIP-depleted, and unfractionated spleen cell suspensions were cultured in agar-medium containing either NIP-coated SRBC or normal SRBC. Table 4 shows the results from one out of two separate experiments. The number of plaque forming colonies of unfractionated spleen cells in agar containing NIP-SRBC was not significantly greater than the number of SRBC-AF-BLC and no lysis of NIP-SRBC was detected in cultures of NIP enriched spleen cells. Apparently, cells which might give rise to NIP-AF-BLC do not possess high affinity receptors for NIP-gelatin allowing enrichment by this method. The fractionation procedure did not affect the frequency of total BL-CFC.

DISCUSSION

The experiments reported here have shown that 0·05–0·25% of B-lymphocyte colonies from normal mouse spleen and lymph nodes are capable of producing lytic plaques when the agar-medium contains SRBC, and complement is added to the colony

Table 2. Effect of SRBC and 2-ME in liquid spleen cell cultures prior to agar seeding. The SRBC-AF-BLC were counted in twenty replicate cultures with 25,000 cells per dish. Total BLC are mean values of four replicate cultures, ±SD values are included

	−2-ME −SRBC		+2-ME −SRBC		−2-ME +SRBC		+2-ME +SRBC	
Day of liquid culture	SRBC-AF-BLC per 10^6 cells	BLC per 25,000 cells	SRBC-AF-BLC per 10^6 cells	BLC per 25,000 cells	SRBC-AF-BLC per 10^6 cells	BLC per 25,000 cells	SRBC-AF-BLC per 10^6 cells	BLC per 25,000 cells
1	0	270 ± 50	23 ± 5	305 ± 35	17 ± 2	290 ± 15	13 ± 5	570 ± 105
2	50 ± 5	600 ± 40	33 ± 5	580 ± 20	40 ± 5	620 ± 75	13 ± 5	620 ± 55
3	3 ± 2	245 ± 75	37 ± 7	380 ± 42	22 ± 4	530 ± 115	7 ± 2	320 ± 95
4	0	135 ± 70	10 ± 2	185 ± 20	3 ± 2	130 ± 10	3 ± 2	190 ± 15

Table 3. Effect on cell and colony numbers of treatment of pool of adult CBA spleen cells with anti-Ig serum plus complement or normal serum plus complement. The experiment was performed twice with similar results

	Number of cells and colonies after treatment		
	Normal serum + complement	Anti-Ig serum + complement	% Reduction
Total number of cells recovered after treatment	$3{\cdot}1 \times 10^6$	$1{\cdot}5 \times 10^6$	50
Cells cultured*	600,000	600,000	—
BLC	12,000	800	93
SRBC-AF-BLC	16	1	94

* Cells were assayed in twenty-four replicate cultures with 25,000 cells per dish.

Table 4. Frequencies of total BLC and BLC which lyse NIP-SRBC and SRBC-AF-BLC in a pool of 12 adult CBA spleens enriched or depleted for NIP reactive cells prior to culture. This experiment was performed twice with similar results

Cells	Total BLC/10^6 cells	BLC lysing NIP-SRBC/10^6 cells	SRBC-AF-BLC/10^6 cells
NIP-enriched*	30,000	0	n.t.
NIP-depleted†	27,000	30	24
Unfractionated‡	25,000	17	11

* Total number of cultured cells = 100,000.
† 160,000 cells cultured with NIP-SRBC and 160,000 cells cultured with SRBC.
‡ 10^6 cells cultured with NIP-SRBC and 10^6 cells cultured with SRBC.
n.t.: not tested.

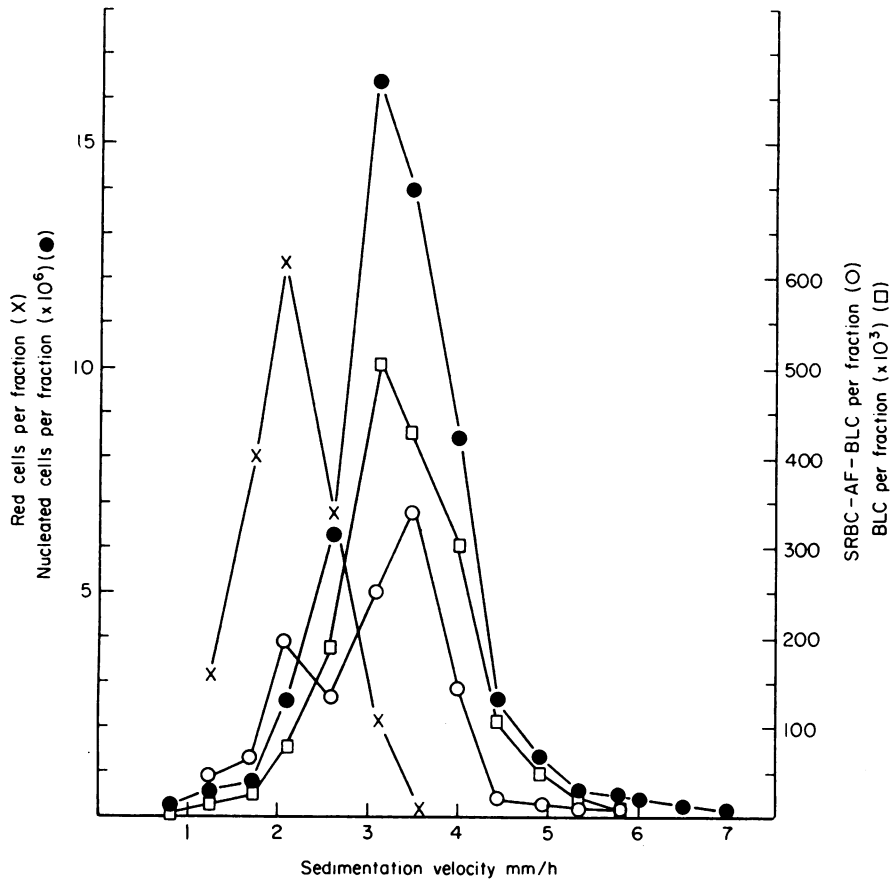

Figure 4. Velocity sedimentation profile of SRBC-AF-BLC-forming cells obtained from a pool of CBA spleen cells. Each point (○) represents colony counts in 24 replicate cultures with 25,000 cells per dish. The profiles for total BLC and for nucleated spleen cells and mouse red blood cells (RBC) are also shown. The curves represent the mean of two separate experiments.

cultures on day 7 of incubation. As SRBC were present throughout the culture period to enhance colony growth (Metcalf *et al.*, 1975) it is not clear whether SRBC-AF-BLC arose as a result of polyclonal stimulating agents in the agar-medium, e.g. agar mitogens (Kincade, Ralph & Moore, 1976), 2-ME, and foetal calf serum, or as a result of antigenic stimulation. Individual colony cells from SRBC-AF-BLC were shown to produce anti-SRBC antibodies of the IgM class, but no IgG was detected. As some BLC cells have been shown to bear surface IgG (Metcalf *et al.*, 1975; Claësson & Metcalf, 1977), it is possible that improved culture conditions allowing later assay, or the addition of factors derived from helper T lymphocytes, might enable BLC to produce IgG.

Specific immunization *in vivo* and *in vitro* had little or no effect on the frequency of SRBC-AF-BLC, whereas the number of specific plaque-forming cells increases by these procedures (Jerne & Nordin, 1963). This finding indicates that SRBC-AF-BLC precursors are not memory cells or specifically activated cells.

On the other hand, the present data show that the vast majority of cells forming SRBC-AF-BLC have surface Ig densities high enough to be killed by anti-Ig plus complement treatment.

The rate of sedimentation (3·5 mm/h) of SRBC-AF-BLC precursors coincided with that of total BLC and total nucleated spleen cells and indicates that the majority of these cells are small lymphocytes.

Separate experiments (Howard *et al.*, 1977) have suggested that the BLC precursor is a mature virgin B cell. The results discussed above, plus the observation that the frequency of SRBC-AF-BLC is low in neonatal spleen and high in spleens from germ-free mice, are consistent with the hypothesis that the cells which give rise to SRBC-AF-BLC are mature virgin B cells; for example, the direct progenitor B cell in the Shortman and Howard model for B lymphocyte differentiation.*

Years ago Robinson *et al.* (1967) showed that spleen cells from unprimed mice when incubated for 3 days in agar medium containing SRBC formed complement dependent plaques, the frequency being about five plaques per 10^6 cells plated. Furthermore, cell division seemed to be necessary for plaque formation. These primary antibody producing spleen cells might be identical to the SRBC-AF-BLC-forming cells described in the present study although the distribution, frequency and potential for proliferation apparently are more restricted for the first category compared to cells giving rise to SRBC-AF-BLC.

It has been demonstrated that spleen cells enriched for hapten (NIP)-binding cells (Haas & Layton, 1975) generate correspondingly elevated proportions of BLC composed of lymphocytes which will bind that hapten, irrespective of the presence of hapten in the culture dish (Metcalf *et al.*, 1975). In the present study, NIP-specific cells were enriched by the same method and BLC grown in the presence of NIP-SRBC. No lysis of NIP-SRBC was found in NIP enriched spleen cells cultured in agar medium although the NIP-specific cells contained the same frequency of BL-CFC as unfractionated spleen, and have previously been shown to produce clones of anti-NIP antibody-forming cells in a liquid microculture system at a frequency of 1 in 40 (Nossal & Pike, 1976). Either the assay system was not capable of detecting anti-NIP antibody formation, or NIP antibody producing BLC are a special subset of BLC arising from cells which could not bind to NIP-gelatin dishes, perhaps because of low surface Ig density. If the latter case is true, this result is in line with the suggestion that specific-AF-BLC belong to an immature B cell category (Osmond & Nossal, 1974; Strober, 1975; Miller, Gorczynski, Lafleur, McDonald & Phillips, 1975).

An alternative explanation for failure to detect NIP antibody forming BLC in cultures of NIP-enriched cells is that specific antibody released early in the culture period could form complexes with antigen (NIP-SRBC) leading to inhibition of differentiation of colonies with a potential for specific antibody production. Such antigen–antibody complex-mediated inhibition of precursor differentiation has been shown in the microculture system mentioned above (Stocker, 1976).

The present assay system for detection of cells giving rise to specific antibody-forming colonies can theoretically be applied to any antigen which can be coated on a red cell indicator. This system for amplifying a given specific B cell function on a clonal basis might be a helpful tool in the study of processes regulating B lymphocyte proliferation and differentiation.

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