PARTICIPATION OF THREE CELL TYPES IN THE ANTI-SHEEP RED BLOOD CELL RESPONSE IN VITRO

Separation of Antigen-Reactive Cells from the Precursors of Antibody-Forming Cells*

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Recent studies by Claman et al. (1), Davies and collaborators (2) and Mitchell and Miller (3) have established that two cell types are involved in the synthesis of anti-sheep red blood cell (SRBC)[1] antibodies in vivo. One of these cells is thymus-derived and the other bone marrow-derived and thymus-independent. It is the latter which gives rise to antibody-forming cells (AFC). Both cell types were shown to be radiosensitive.

In vitro, two cell types, a glass-adherent and a nonadherent population, were separated and shown to be required for an anti-SRBC response (4–6); the former cell was found to be radioresistant. A correlation of the in vitro and in vivo results and further elucidation of the number and nature of the participating cells depends on the development of methods of fractionation capable of separating the different cell types involved. Haskill et al. (7) using a method of equilibrium density centrifugation obtained two main populations of cells. Analysis of their data suggested that three cell types participate in the in vitro response in accordance with the suggestion of Mosier and Copplestone (8).

In the present investigation three cell types were identified and separated. One of these was shown to be a thymus-dependent, radiosensitive cell, the second a thymus-independent radiosensitive cell, the precursor of AFC, and the third a radioresistant cell.

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; ARC, antigen-reactive cells; CBAf, filtered CBA cells; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; PFC, plaque-forming cells; RFC, rosette-forming cells; S-MEM, minimum essential medium for suspension culture; SRBC, sheep red blood cells.

Materials and Methods

Animals and Antigens Used.—10–16-wk old male and female CBA (Jackson Laboratories, Bar Harbor, Maine) and C57BL mice (McIntyre Animal Center, McGill University, Montreal) were used throughout. Sheep red cells (SRBC) stored at 4°C in Alsever's solution were obtained biweekly from the Department of Microbiology and Immunology, McGill University.

Biological Reagents.—Hanks' balanced salt solution (HBSS), minimum essential medium for suspension culture (S-MEM), medium CMRL 1066, and medium 199 were obtained from Grand Island Biological Co., Grand Island, N. Y.; fetal calf serum (FCS) and guinea pig complement were obtained from Hyland Laboratories, Los Angeles, Calif.

Preparation of Cell Suspensions.—Mice were killed by cervical dislocation and their removed spleens were placed immediately into ice-cold HBSS. The spleens were minced with scissors and gently pressed through a 50-mesh stainless steel sieve. Further dissociation of the cells was achieved by aspirating the suspension up and down using a Pasteur pipette. The remaining large particles were allowed to sediment out and were discarded, while the suspension of dissociated cells was centrifuged down for 10 min at 900 rpm. The cells were washed once in HBSS and were finally suspended in medium CMRL 1066 containing 20% FCS.

Bone marrow cells were prepared from the femur, tibia, and humerus of mice by extruding the bone marrow plugs with a syringe and a 20 gauge needle into cold medium 199. The cells were suspended using a Pasteur pipette, were washed once, and were resuspended in the same medium.

Sheep red cells were washed three times in 20–30 volumes of HBSS just before use. They were finally suspended in medium CMRL 1066 for injection or addition to the culture. All cell suspensions were maintained at 4°C throughout the period of preparation.

Fractionation.—Cells were "filtered" through columns of cotton wool (India Cotton, gift of Johnson & Johnson, Montreal, Canada) according to a modification of Lamvik's procedure (9). Pyrex glass tubing, 25 cm in length and 1 cm O. D., was filled with 2 g of cotton wool. To ensure even packing, columns were always filled to the same length, i.e. 22–23 cm. The filled columns were sterilized by autoclaving for 30 min at 15 lb pressure.

The filtration was carried out in a room maintained at 37°C. 20–30 ml of cell suspension containing 10×10^6 cells/ml was applied to each column prerinsed with 10 ml of medium. The cells were eluted with 30–40 ml of medium CMRL 1066 containing 10% FCS. The cells were collected by centrifugation and dispersed in the same medium containing 20% FCS.

In some experiments the procedure was slightly modified. Only 10 ml of suspension (100×10^6 cells) was applied to the column. The cells were collected in two fractions; the first fraction comprised the cells which passed through unimpeded in approximately the initial volume applied and a second fraction emerged in the "wash". Portions of the two fractions were recombined and were passed through a second column. The yield obtained from the standard procedure was approximately 30%, that from the modified procedure for the first fraction was 4–6%, for the wash 10%, and for the refiltration 60%.

Isolation of Rosettes.—Rosettes were formed using either C57BL or CBAf preparations in one of two ways. 40 million nucleated spleen cells were mixed with 240 million SRBC in a total volume of 20 ml of CMRL 1066 containing 10% FCS. The cells were kept at 4°C overnight. They were then centrifuged, resuspended gently in 20 ml of phosphate-buffered saline (PBS, pH 7.4) containing 3% FCS. Alternately, the same number of freshly prepared cells were suspended in the latter medium, centrifuged at 500 rpm for 15 min, incubated on ice for 30 min, and then resuspended. One of the preparations was then loaded onto a Staput apparatus made by Johns Glass, Toronto, Canada, to the specifications of Miller and Phillips

(10). The cells were allowed to sediment under gravity at 4°C for 3.5 or 4 hr and were eluted in three fractions: the rosette-containing fraction (sedimenting at a rate of 8 mm/hr or more), an intermediate fraction (5–8 mm/hr), and the rest of the cells containing most of the small lymphocytes and erythrocytes. The cells in each fraction were washed with HBSS containing 10% FCS and dispersed in CMRL medium 1066 containing 20% FCS.

Irradiation.—Mice were irradiated in plastic containers from a cobalt-60 source at a rate of 53 R/min for a total of 750–900 R. They were sacrificed on the day after irradiation. Spleen cell suspensions were irradiated in plastic tubes at a rate of 80 R/min for a total dose of 800 R.

Thymectomy and Repopulation with Isogeneic Bone Marrow Cells.—6–8-wk old mice were thymectomized by the method of Jeejeebhoy (11). 2 wk after thymectomy the mice received a single dose of 900 R of total body irradiation followed immediately by an intravenous injection of 1×10^7 isogeneic bone marrow cells. A minimum of 6 wk were allowed to elapse before these animals were used for experiments.

Cell Culture.—The culture method used was that described by Marbrook (12). The culture vessel consisted of an inner glass tube (I. D. 1.1 cm, length 9 cm) closed at the bottom with a dialysis membrane (Union Carbide Corp., Visking Division, Lindsay, Ontario, Canada) held in place with a ring cut from silicone tubing. This inner tube was inserted into a glass vial (2.7 × 5.6 cm, Packard Instrument Co., Inc., Downers Grove, Ill.) and was covered and held in place by a porous plastic plug (Scientific Products, Evanston, Ill.). The vessels were autoclaved for 30 min at 15 lb of pressure. For culture the outer chamber was filled with 9 ml of medium CMRL 1066 containing 20% FCS; the inner chamber held 1 ml of the same medium containing the spleen cells and 2×10^6 SRBC. The height of the inner tube was adjusted to equalize the fluid level in the two compartments. The cultures were incubated without agitation in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% compressed air for 5 days. Each culture was set up in triplicate. Two samples from each vessel, 0.2 and 0.4 ml, were assayed for plaque-forming cells (PFC) by the method of Jerne and Nordin (13). The number of PFC are expressed as per culture or per million cells cultured; the figures represent averages from triplicate cultures.

Preparation of Isoantisera and Serum Treatment of Plaque-Forming Cells (PFC).—6 wk-old CBA and C57BL mice were given six to eight weekly intraperitoneal injections of 50–100 million cells from pooled thymus, spleen, and lymph nodes of adult C57BL and CBA mice in volumes of 0.2 ml. The hyperimmunized mice were bled 6–8 days after the last injection. The appropriate sera pooled were deactivated by heating them at 56°C for 30 min and were stored in small portions at −20°C. The cytotoxic titer of both serum preparations as well as that of a horse anti-mouse serum used (serum pool B, from the Medical Research Council of Canada) was of the order of 1:640. Primed spleen cells or cultured lymphocytes were treated with antiserum according to the method of Mitchell and Miller (3). To 0.15 ml portions containing 1–3 million cells with approximately 100–500 PFC, 0.05 ml of undiluted guinea pig serum and 0.05 ml of undiluted antiserum or control normal serum were added. The cells were incubated at 37°C for 30 min, then collected by centrifugation. They were washed once in CMRL 1066, resuspended in 0.3 ml of the same medium containing 10% FCS, and plated on agar for the development of plaques.

RESULTS

The efficiency of the in vitro culture system using CBA spleen cells is demonstrated in Fig. 1. The number of plaque-forming cells (PFC) obtained in vitro is comparable to that obtained after immunization in vivo. The optimal cell concentration (Fig. 2) was found to be between 15 and 20 million cells per culture, and this concentration was used throughout most of the following ex-

periments. In contrast to these results obtained with cells of CBA mice, cells of C57BL mice gave no response in vitro under the culture conditions used. Similarly, CBA spleen cells which were passed through columns of cotton wool, referred to as filtered cells (CBAf), gave a much reduced response or no response at all when cultured with antigen. However, CBA filtered cells cultured together with C57BL spleen cells gave a response which exceeded that given by the CBA unfractionated cells cultured alone (Table I). The number of both the

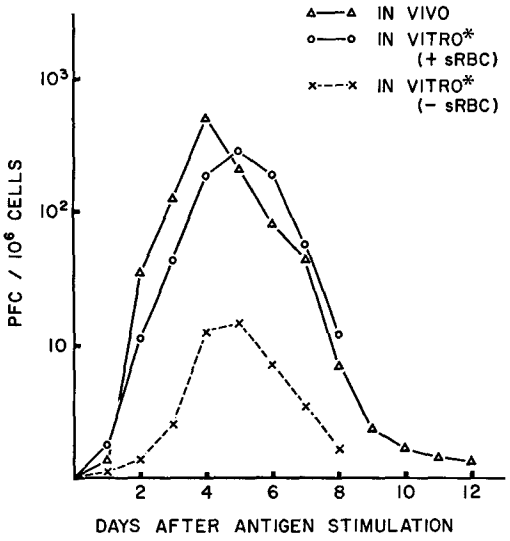

FIG. 1. PFC response of normal CBA mice to SRBC. Each point is an average of the results obtained in six plates (in vivo) or in three parallel cultures (in vitro). Each experiment was carried out twice.

* 15×10^6 spleen cells were cultured; the PFC are expressed as per 10^6 cells recovered.

CBAf cells (Fig. 3) and that of the C57BL cells (Fig. 4) was rate limiting in these combined cultures and a ratio of 1:1 was found to be optimal.

The Role of C57BL Spleen Cells in the Combined Cultures.—Adherent cells: Since filtration through cotton wool is known to remove adherent cells required for the anti-SRBC antibody response in vitro, the role of C57BL cells may be to restore these cells to the CBAf fraction. To examine whether this is the role and the only role of the C57BL cells, adherent cells of another source were used. As can be seen in Table II, when CBA filtered cells were cultured with 5×10^6 CBA irradiated cells as a source of adherent cells (14, 15), the response was only partially restored and doubling the number of the irradiated cells added (Table III) did not result in a further increase. These results suggest that the C57BL cells contribute to the combined culture a cell or function deficient in

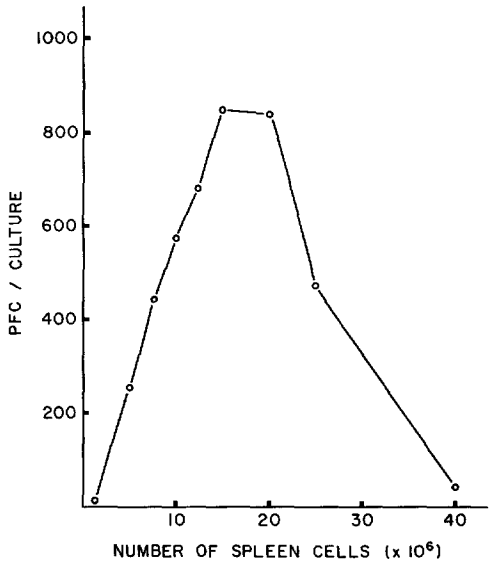

FIG. 2. The effect of cell concentration on the PFC response. Each point is the average obtained from three parallel cultures. The experiment was carried out three times.

TABLE I

Antibody Synthesis in Cultures Containing CBA, CBAf, C57BL, and Mixtures of CBAf and C57BL Spleen Cells

Cells in culture*	PFC/culture‡		
	Exp. 1	Exp. 2	Exp. 3
20×10^6 CBA	907	824	845
10×10^6 CBAf	69	58	12
20×10^6 C57BL	7	9	5
5×10^6 C57BL	—	—	—
2×10^6 C57BL	—	—	—
10×10^6 CBAf + 2×10^6 C57BL	739	961	870
10×10^6 CBAf + 5×10^6 C57BL	1202	578	1025

* CBAf refers to spleen cells from CBA mice filtered through columns of cotton wool. Unless otherwise indicated, CBA and C57BL refer to untreated, nonfractionated spleen cells from CBA and C57BL mice respectively.

‡ The antigen concentration used was 2×10^6 SRBC per culture and the number of plaque-forming cells (PFC) was determined on the 5th day. Each experiment was done with cultures in triplicate. The PFC indicated are averages from the three cultures; the maximum variation within each triplicate was $\pm 10\%$.

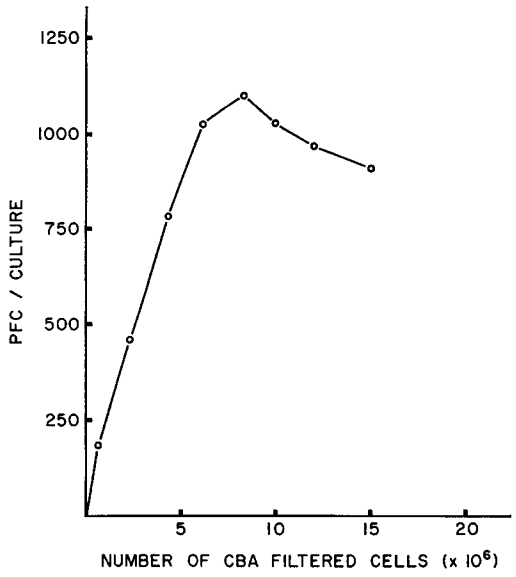

FIG. 3. Kinetics of the PFC response in the combined cultures. 7.5 × 10^6 C57BL cells and 2 × 10^6 SRBC were added to each culture of CBA filtered cells. Each point is an average from three parallel cultures. The experiment was repeated three times.

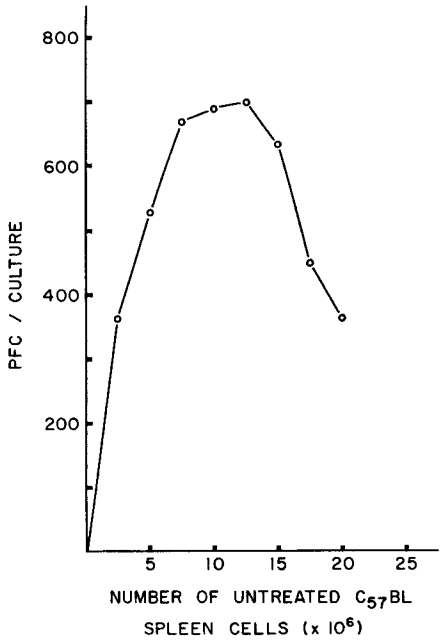

FIG. 4. Kinetics of the PFC response in the combined cultures. 2 × 10^6 CBA filtered cells and 2 × 10^6 SRBC were added to each culture of C57BL cells. Each point is an average from three parallel cultures. The experiment was repeated three times.

TABLE II

Antibody Synthesis in Cultures Containing CBA Filtered and 5 × 10^6 CBA Irradiated Cells

Cells in culture*	PFC/culture			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
15 × 10^6 CBAir	—	—	—	—
5 × 10^6 CBAf	5	—	12	13
10 × 10^6 CBAf	17	5	52	38
15 × 10^6 CBAf	80	16	73	61
5 × 10^6 CBAf + 5 × 10^6 CBAir	60	17	85	46
10 × 10^6 CBAf + 5 × 10^6 CBAir	113	35	182	108
15 × 10^6 CBAf + 5 × 10^6 CBAir	244	85	297	197
20 × 10^6 CBA	1168	533	1231	988

* CBAir refers to spleen cells taken from mice 1 day after receiving 800 R total body irradiation. Other experimental conditions are as described in the caption to Table I.

TABLE III

Antibody Synthesis in Cultures Containing CBA Filtered and 10 × 10^6 CBA Irradiated Cells

Cells in culture*	PFC/culture		
	Exp. 1	Exp. 2	Exp. 3
10 × 10^6 CBAir	—	—	—
15 × 10^6 CBAf	69	38	75
5 × 10^6 CBAf + 10 × 10^6 CBAir	39	45	67
10 × 10^6 CBAf + 10 × 10^6 CBAir	77	76	117
15 × 10^6 CBAf + 10 × 10^6 CBAir	211	136	229
15 × 10^6 CBA	742	511	789

* CBAir refers to spleen cells taken from mice 1 day after 800 R total body irradiation. Other experimental conditions are as described in the caption to Table I.

TABLE IV

Antibody Synthesis in Cultures Containing CBA Filtered and Suboptimal Concentrations of CBA Normal Spleen Cells

Cells in culture	PFC/culture		
	Exp. 1	Exp. 2	Exp. 3
10 × 10^6 CBAf	30	12	58
2 × 10^6 CBA	—	—	—
5 × 10^6 CBA	39	10	121
2 × 10^6 CBA + 10 × 10^6 CBAf	324	289	252
5 × 10^6 CBA + 10 × 10^6 CBAf	566	455	549
20 × 10^6 CBA	768	845	824

The experimental conditions are as described in the caption to Table I.

both the CBA filtered and in the irradiated population. That such a cell, a third cell type, is required for the anti-SRBC response is also suggested by the results illustrated in Table IV. Suboptimal concentration of unfractionated CBA spleen cells, unable to elicit a response by themselves, gave a synergistic response with CBA filtered cells.

TABLE V

PFC Response of Spleen Cells of CBA and C57BL Mice Immunized In Vivo, Incubation with Antisera

Spleen cell donor		PFC remaining after treatment with*							
		Normal mouse serum		Horse anti-mouse serum		Anti-CBA serum		Anti-C57BL serum	
	Exp.	No.	%	No.	%	No.	%	No.	%
CBA	1	274	100	36	13	13	5	242	90
	2	351	100	42	12	18	6	323	92
C57BL	1	616	100	86	14	562	91	81	13
	2	483	100	63	13	434	92	53	11

* Samples containing 1–3 × 10^6 CBA or C57BL immune spleen cells in 0.15 ml were incubated with 0.05 ml of guinea pig complement and 0.05 ml of undiluted antisera for 30 min at 37°C. The cells were washed and assayed for remaining PFC.

TABLE VI

The Origin of PFC from CBAf–C57BL Combined Cultures

Cells in culture*		PFC remaining after treatment with							
		Normal mouse serum		Horse anti-mouse serum		Anti-CBA serum		Anti-C57BL serum	
	Exp.	No.	%	No.	%	No.	%	No.	%
15 × 10^6 CBA	1	165	100	5	3	11	7	171	100
	2	203	100	3	1	12	6	200	99
7.5 × 10^6 C57BL + 7.5 × 10^6 CBAf	1	358	100	22	6	269	75	140	39
	2	271	100	19	7	214	78	89	32

* The cells cultured were either CBA alone or CBA filtered cells and C57BL spleen cells combined. The conditions for the treatment with the antisera were the same as in Table V.

Antibody-forming cells: In searching for a second function for the C57BL cells in the CBAf–C57BL combined cultures, the origin of the antibody-forming cells was investigated. Portions of cells recovered from CBAf–C57BL combined cultures were exposed to the action of either an anti-C57BL or an anti-CBA serum and complement. The cells remaining after this treatment were developed for PFC. The specificity of the antisera used and the adequacy of the treatment was ascertained using cells from animals primed in vivo (Table V).

The anti-CBA serum caused lysis of 95% of CBA target cells and affected only 9% of C57BL cells, whereas the anti-C57BL serum inhibited 87% of the C57BL PFC and only 8% of the CBA cells. The PFC remaining after treatment with the specific sera were comparable to the number of PFC escaping treatment with a horse anti-mouse serum. Results obtained using the same sera on cells primed in vitro are shown in Table VI. Whereas the anti-C57BL serum did not

TABLE VII

The Origin of PFC from CBAf–C57BL Combined Cultures

Cells in culture*	PFC remaining after treatment with									
7.5×10^6 C57BL combined with	Normal mouse serum		Horse anti-mouse serum		Anti-C57BL + anti-CBA serum		Anti-CBA serum		Anti-C57BL serum	
	No.	%	No.	%	No.	%	No.	%	No.	%
CBAf 0.5×10^6	249	100	20	8	26	10	202	80	61	24
CBAf 2×10^6	314	100	25	7	43	13	252	75	99	30
CBA fraction I 0.5×10^6	174	100	14	7	17	9	160	87	34	18
CBA fraction I 2×10^6	265	100	18	4	29	11	226	88	40	16
CBA fraction II 2×10^6	95	100	13	13	12	12	68	66	24	24
CBA fraction III 0.5×10^6	215	100	15	7	27	13	187	89	21	10
CBA fraction III 2×10^6	282	100	21	8	25	9	267	92	33	12

* 7.5×10^6 C57BL spleen cells were cultured together with 0.5 or 2×10^6 CBA cells passed through columns of cotton wool according to the routine (CBAf) or to a modified procedure. In the modified procedure 100×10^6 cells were applied on each column in a volume of 10 ml. The cells were collected in two fractions: fraction I collected first consists of the cells which passed through the column unimpeded; cells in fraction II were collected subsequently in the wash. Cells in fraction III were filtered twice. Portions of cells from each culture were incubated with antisera and complement. The numbers recorded are the PFC remaining after serum treatment.

inhibit the PFC from cultures of CBA cells, the same serum inhibited 61–68% of the PFC from the combined cultures, suggesting that the majority of PFC in these cultures were derived from the C57BL population. To find out whether the contribution of the CBAf cells to the PFC population was an inherent property of that fraction or whether it only reflected a residual activity due to inadequate fractionation, the isoantiserum treatment was repeated on combined cultures established with more thoroughly fractionated cells (Table VII). The results obtained indicate that the more thorough the filtration, the smaller the contribution of the CBA population to the PFC. Thus, the number of PFC

which may have been derived from CBA prepared in the usual manner was of the order of 25–30%; this was similar in fraction II, in the modified procedure, representing cells retarded and eluted from the columns in the wash. In cultures where fraction I cells, which passed through the columns unimpeded, were used the proportion of CBA plaques dropped to 16–18%. The best preparation was obtained with cells filtered twice (fraction III). In this fraction the PFC of CBA origin was 10–12%, a figure not exceeding and not distinguishable from controls, i.e., PFC remaining after treatment with a combination of both anti-CBA and anti-C57BL sera or with a horse anti-mouse serum. Thus it may be concluded that in the CBAf–C57BL combined cultures the majority if not all of the precursors of PFC are derived from the C57BL population.

TABLE VIII

Antibody Synthesis in Cultures Containing CBA filtered cells, CBA irradiated cells and Rosettes made with C57BL Spleen Cells and SRBC

Cells in culture*	PFC/culture	
	Exp. 1	Exp. 2
CBAir + C57N	14	4
CBAf + CBAir	0	30
CBAf + C57 rosettes	36	75
CBAf + CBAir + C57 rosettes	256	198

* The rosettes were prepared by incubation of 40×10^6 C57BL spleen cells with 240×10^6 SRBC. The rosette-containing fraction used was separated in a Staput apparatus; cells derived from 13 and 7×10^6 spleen cells were added to each culture in the first and second experiment respectively. The concentration of CBA filtered cells (CBAf) was 5×10^6 and that of the irradiated cells (CBAir) was 3×10^6 per culture. The culture conditions used were as described in the caption to Table I.

The experiments described in the foregoing sections implied that the C57BL cells contribute two cell types to the combined cultures, namely an adherent cell and the precursor of PFC. In the following experiments an attempt was made to separate the two cell types and thus to demonstrate directly this dual contribution (Table VIII). A fraction, containing predominantly rosettes formed between C57BL spleen cells and SRBC, was isolated. These rosettes and CBA filtered cells were cultured together with or without the addition of CBA irradiated cells as a source of adherent cells (Table VIII). As can be seen in two experiments 36 and 75 PFC were detected in cultures grown in the absence of adherent cells; the addition of CBA irradiated cells raised the number of PFC to 256 and 198 respectively, demonstrating the contribution of adherent cells. The participation of the rosettes, presumably containing the precursors of AFC, was shown by the virtual absence of PFC (0 and 30 PFC respectively) containing only CBA filtered and CBA irradiated cells.

The Role of the CBAf Cells in the Combined Cultures.—Three aspects of the contribution of the CBAf cell were examined. These were its radiosensitivity, its thymus-dependence, and its relation to rosette-forming cells (RFC). Results in Table IX indicate that the CBAf cell is radiosensitive. To examine its thymus

TABLE IX

The Effect of X-irradiation on CBA Filtered Cells

Cells in culture*	PFC/culture	
	Exp. 1	Exp. 2
15×10^6 C57BL	8	17
15×10^6 CBAf, irradiated	—	—
2×10^6 CBAf + 5×10^6 C57BL	472	
2×10^6 CBAf + 10×10^6 C57BL	739	
2×10^6 CBAf, irradiated + 5×10^6 C57BL	5	
2×10^6 CBAf, irradiated + 10×10^6 C57BL	13	
3×10^6 CBAf + 5×10^6 C57BL		629
3×10^6 CBAf + 10×10^6 C57BL		851
3×10^6 CBAf, irradiated + 5×10^6 C57BL		9
3×10^6 CBAf, irradiated + 10×10^6 C57BL		74

* The filtered, irradiated cells were prepared from spleens of mice given 800 R total body X-irradiation 1 day earlier. Other experimental conditions are described in the caption to Table I.

TABLE X

The Effect of Thymectomy on Antibody Response in C57BL–CBAf Combined Cultures

Cells in culture	PFC/culture	
	Exp. 1	Exp. 2
15×10^6 CBA	1038	977
15×10^6 CBA Tx*	25	32
15×10^6 C57BL	—	—
15×10^6 C57BL Tx	—	—
10×10^6 CBAf	—	—
7.5×10^6 C57BL + 7.5×10^6 CBAf Tx	53	10
7.5×10^6 C57BL, Tx + 7.5×10^6 CBAf	832	1030
7.5×10^6 C57BL + 7.5×10^6 CBAf	824	992

* Tx refers to animals thymectomized, irradiated, and repopulated with syngeneic bone marrow. Culture conditions were as described in the caption to Table I.

dependence, and that of the C57BL spleen cell, combined cultures of CBAf and C57BL spleen cells were established in which one of the cell populations was obtained from a thymectomized, X-irradiated, bone marrow-repopulated mouse. The results obtained indicate (Table X) that the contribution of the CBAf, unlike that of the C57BL cells, is dependent on the thymus.

Results presented in Table VIII have indicated that rosette-forming cells of

the C57BL spleen constitute one of the cell types contributed by the C57BL population to the combined cultures. Similar experiments in which RFC fractions prepared from the CBAf population were cultured together with C57BL cells were uniformly negative, indicating that if such rosettes were formed, they did not comprise the CBAf cell essential for the antibody response.

DISCUSSION

The main contribution of the present study is the direct demonstration of the participation of three components in anti-SRBC antibody synthesis in vitro. This demonstration depended primarily on a separation of the precursors of antibody-forming cells from antigen-reactive cells. Although an enrichment of antibody-forming cells from immune animals has been achieved on glass bead columns (16), the separation of the precursors of AFC of normal unimmunized mice on cotton wool was unexpected. That such a separation could be obtained implies that the precursors of AFC differ from ARC in physical characteristics such as size and/or surface properties. Preliminary experiments indicate that this difference between the AFC and ARC also exists in the anti-chicken RBC system. The general applicability of this observation for other antibody–antigen systems remains to be verified.

The second observation obtained in several laboratories, basic to this study, was that spleen cells of C57BL mice do not respond to SRBC in vitro. The present investigation has shown that this lack of response is due to a "defect" at the level of the ARC. The nature of this defect is not known except for the finding that it can be corrected by incorporating L-asparagine into the culture medium.[2] It may be argued that the nature of the contribution of the CBA cells to the CBAf-C57BL combined cultures is to supply L-asparagine, thus allowing the C57BL cells to respond. This interpretation however is unlikely in view of the lack of response in CBAf–C57BL combined cultures when the CBA cells are derived from a thymectomized, irradiated, bone marrow-repopulated animal. Using C57BL spleen cells in culture in the absence of added L-asparagine provided a preparation of bone marrow-derived cells functionally devoid of thymus-derived cells. The contribution of the C57BL cells to the combined cultures was further dissected by separating the AFC as rosettes from the radioresistant "adherent" cells.

The nature and identity of rosette-forming cells in normal and immunized mice is still in dispute. Brody (17) reported that RFC separated from the spleen of normal mice were of bone marrow origin. Greaves and Moller claimed that at least in immunized animals a large proportion of RFC are thymus derived (18). In the present investigation RFC separated from the spleen of C57BL mice were capable of antibody formation in the presence of CBAf thymus-derived cells, thus corroborating the results of Brody. Conversely thymus-derived

[2] Osoba, D. Personal communication.

cells required in the CBAf–C57BL cultures could not be supplied in the form of rosettes.

The results described in this communication are in agreement with previous suggestions that three cell types are involved in the in vitro antibody response to SRBC (7, 8). They also corroborate the results of Mosier et al. (19) and those of Munro and Hunter (20) showing that the in vitro antibody response is thymus dependent. In addition, the present findings in vitro extend the results of Mitchell and Miller obtained in vivo (3); they indicate that the antibody-forming cell is not derived from the thymus-dependent antigen-reactive cell.

SUMMARY

Spleen cells of unprimed CBA mice were shown to produce anti-sheep red blood cell antibodies comparable in amount in vivo and in vitro. Under identical culture conditions spleen cells of C57BL mice did not respond. CBA spleen cells, passed through columns of cotton wool (CBAf), were equally inactive in vitro. However combined cultures containing both CBAf and C57BL cells yielded as many or more plaque-forming cells than the same number of unfractionated CBA spleen cells. Analysis of the contribution of each cell population to the synthesis of antibody in the combined cultures has disclosed the participation of three cell types. A thymus-dependent, radiosensitive cell was derived from the CBAf population, while the C57BL was the source of the precursor of the antibody-forming cell and of a radioresistant cell. The latter two were partially separated in a Staput apparatus.

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