SOME PHYSICAL AND RADIOBIOLOGICAL PROPERTIES OF IMMUNOLOGICALLY REACTIVE MOUSE SPLEEN CELLS*

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The generation of hemolysin-forming cells by mouse spleen cells in culture may require the participation of three classes of cells (1–3). This conclusion is suggested by the results of experiments in which spleen cells were separated on the basis of differences in their capacity to adhere to plastic (1, 2), in their density (3), and in their radiation sensitivity (1, 3–6).

In the present experiments, the procedures of velocity sedimentation (7), rosette formation (8, 9), and irradiation were used to define some of the properties of each class of cells. Velocity sedimentation separates cells according to their size. Since rosette formation, in effect, alters the size of the cells which bind erythrocytes to their surface, such cells can be separated from the remaining spleen cells by velocity sedimentation. In some experiments, cells separated by these procedures were heavily irradiated to determine the effect of ionizing radiation on their subsequent capacity to participate in the production of hemolysin-forming cells.

The results suggest that three classes of cells are required for the production of hemolysin-forming cells in culture. One class of cells has the capacity to form rosettes. Another class can still participate in the immune response after exposure to a large dose of gamma radiation. The existence of a third class, having different properties than the above two classes, was deduced from the results of mixing experiments.

Materials and Methods

Cell Suspensions.—Spleen cells were obtained from 10–12 wk old normal CBA mice (Jackson Laboratories, Bar Harbor, Me.) not previously immunized with foreign erythrocytes. The mice were killed by cervical dislocation, and the spleens removed aseptically. The capsule was slit along its longitudinal axis, and the cells gently expressed into cold phosphate buffered saline (PBS). Clumps of cells and the splenic capsule with adhering cells were placed in a small flask and stirred on a magnetic stirrer for 30 min. Following this procedure the cells were allowed to sediment for 2–3 min. The suspended cells were removed, leaving behind the remnants of the splenic capsule. Further separation of large clumps of cells was achieved by passing the cell suspension through a fused glass capillary array (mosaic filter, pore size, 37 μ [Bendix, Mosaic Fabrications Division, Sturbridge, Mass.]). After filtration, the cell suspension consisted almost entirely of single cells, but a few clumps containing up to 15 cells were still present. When tested by the fluorescence method of Rotman and Papermaster (10), more than

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95% of the cells fluoresced after a 30 min exposure to a 1:10,000 concentration of fluorescene diacetate.

Sedimentation Procedure.—The method of velocity sedimentation has been described by Miller and Phillips (7). Two sedimentation vessels, having diameters of 17 and 21.5 cm, were used depending upon the number of cells required for a given experiment. An appropriate volume of PBS was loaded first, followed by the cell suspension made up to a concentration of 5×10^6–10^7 cells per ml in 3% fetal calf serum (Flow Laboratories, Rockville, Md.) and PBS. The total volume of cells loaded was adjusted to produce a band exactly 2 mm in depth regardless of the size of the sedimentation vessel. A small volume of 5% fetal calf serum and finally a nonlinear gradient of 15–30% fetal calf serum in PBS were loaded under the cell suspension. The cells were allowed to sediment under the force of unit gravity at 4°C for an appropriate period of time, usually 2.5–4 hr. Fractions were collected by draining the contents of the sedimentation vessel into centrifuge tubes. The number of cells in each fraction was determined by counting an aliquot of each fraction in an electronic cell counter having an aperture size of 100 μ (Coulter Counter Model F, Coulter Electronics Inc., Hialeah, Fla.). To obtain nucleated cell counts, erythrocytes were lysed by the addition of Zap-isoton (Coulter Diagnostics, Inc., Hialeah, Fla.) to each aliquot.

A typical profile of the cell distribution obtained after sedimenting normal spleen cells is shown in Fig. 1. It was found in repeated experiments that this profile was highly reproducible, the peak sedimentation velocity $\pm$ 1 SE for nucleated spleen cells being 3.3 $\pm$ 0.2 mm per hr, and for mouse erythrocytes, 2.0 $\pm$ 0.15 mm per hr.

Erythrocytes.—Sheep erythrocytes (SRBC) were obtained weekly from the same sheep (Woodlyn Farms, Guelph, Ont.). Chicken erythrocytes (CRBC) were obtained each week by bleeding separate chickens from the same flock of White Leghorns. Both kinds of erythrocytes were stored in citrate solution at 4°C. Prior to use in any procedure the erythrocytes were washed three times in PBS, care being taken to remove the buffy coat.

Formation of Rosettes.—A modification of the rosette-forming procedure described by Edwards et al. (11) was used. Spleen cells were washed once, and a suspension of washed SRBC or CRBC was added so that the ratio of RBC to nucleated cells was 8:1. The final concentration of nucleated cells was adjusted to 5×10^6 per ml. This suspension was spun in a refrigerated centrifuge at 100 *g* for 15 min. The tubes were left at 4°C for a further 30 min. The cells were resuspended gently, an aliquot was removed, and the number of rosettes counted in a modified Howard counting chamber (C. A. Hausser and Son, Philadelphia, Pa.). Only spherical structures consisting of red cells resembling a raspberry were counted as rosettes. When the RBC forming these structures were lysed with dilute acetic acid they were found to contain a single nucleated cell. Using this method, the number of rosettes usually varied from 150 to 300 per 10^6 nucleated spleen cells.

Irradiation of Cells.—Suspensions of spleen cells to be used as a source of irradiated cells were made up to a concentration of 5×10^7–10^8 cells per ml and irradiated with a dose of 1500 rads in a ^{137}Cs irradiator (12).

Culture Method.—A modification of the culture method developed by Marbrook (13) was used and has been described in previous publications (5, 6). The culture chamber consists of an inner glass cylinder, one end of which is covered with a dialysis membrane. Cells in culture medium are placed in the cylinder and settle on the dialysis membrane. The cylinder is immersed in a glass vial of culture medium. Modifications consist of using pooled human serum (Philadelphia Blood Supply, Philadelphia, Pa.) instead of fetal calf serum. Prior to use in the cultures the human serum was heated to 56°C for 30 min and filtered. In addition, the size of the inner cylinder was scaled down to give a surface area of 47 mm^2. The cells were cultured in a volume of 0.5 ml, containing tissue culture medium CMRL 1066 (14), 20% pooled human serum, and 20 μg per ml of L-asparagine. The outer vial contained 4.5 ml of CMRL 1066 and L-asparagine, but no human serum.

The peak plaque-forming cell (PFC) response, assayed by the method of Jerne et al. (15), occurs on the 4th day (5). Less than 4×10^6 CBA spleen cells, cultured alone, fail to produce a PFC response. However, when from 10^5 to 4×10^6 normal spleen cells are mixed with 10^7 heavily irradiated spleen cells (1500 rads) PFC responses occur regularly. The number of PFC produced under these conditions is linearly related to the number of normal spleen cells cultured (5, and unpublished observations). The number of "background" PFC produced in the absence of xenogeneic RBC is always less than 5% of the number produced when RBC are present in the cultures.

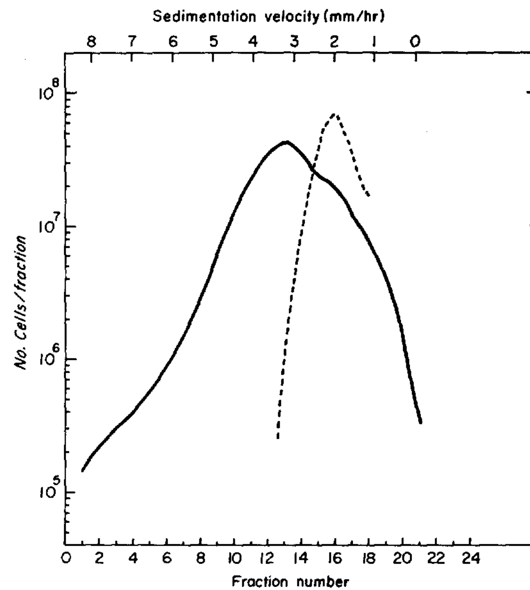

FIG. 1. Sedimentation profile of normal mouse spleen cells. The peak sedimentation velocities of nucleated spleen cells (——) and erythrocytes (- - - -) are 3.2 and 2.0 mm per hr, respectively.

In most of the experiments reported here, cells subjected to sedimentation were cultured in the following manner. All of the cells available in each fraction were used for culture. Fractions containing from 2×10^5 to 2×10^6 cells were divided in half; fractions containing more than 2×10^6 cells were divided into aliquots of 10^6 cells each. These cells were cultured with 10^7 irradiated spleen cells and 2×10^6 SRBC or CRBC. Control cultures consisted of cells obtained from the same pool of cells as were those subjected to sedimentation. The control cells were kept at 4°C and 10^6 cells were placed into culture with 10^7 irradiated spleen cells and 2×10^6 RBC at the same time as were the cells subjected to sedimentation. Any changes in experimental design from the one just described will be given in the Results section.

To construct a profile of the PFC response produced by cells subjected to sedimentation, the number of PFC produced in the cultures from a given fraction were added together and the total PFC in each fraction plotted on a graph. An estimate of the amount of PFC-activity recovered in the sedimented fractions was obtained by adding together all of the PFC produced by the cells in all of the fractions and comparing this number to the number of PFC

produced by an equal number of unsedimented, control cells. These numbers are expressed as the average number of PFC per 10^6 cultured cells in the Results. Since the "background" PFC responses were very small, they were not subtracted from the responses obtained in the presence of RBC.

RESULTS

Sedimentation Profile of the PFC Response by Fractions of Sedimented Spleen Cells.—The object of these experiments was to construct the sedimentation profile of spleen cells capable of giving rise to PFC responses when cultured with heavily irradiated spleen cells and SRBC. The cells in fractions sedimenting from 0.75 to 7 mm per hr were cultured in the presence of irradiated spleen cells and SRBC. In each of seven separate experiments, more than 67% of the total PFC response was obtained in fractions sedimenting at 3–4 mm per hr. The peak response was present in fractions sedimenting at 3.6 ± 0.2 mm per hr. A typical profile of the PFC response obtained in these experiments is shown in Fig. 2 *a*.

When the total PFC response produced by all of the sedimented cells was compared to the PFC response produced by equal numbers of unsedimented control cells, it was found that all of the expected PFC-activity was recovered in the sedimented fractions (Table I).

Sedimentation Profile of Heavily Irradiated Spleen Cells Capable of Facilitating PFC Responses by a Small Number of Normal Spleen Cells.—In previous experiments (3–6) it has been shown that the spleen and lymph nodes contain a class of cells capable of facilitating PFC responses by a small number of normal spleen cells. Radiation survival studies of this facilitative activity showed that it was diminished only after doses in excess of 2.5 krads were given to the spleen cells ($D_{37} = 4.5$ krads) (6). This result suggested that cells capable of facilitating PFC responses did not require a capacity for extensive proliferation to retain their facilitative activity. For the sake of brevity this class of cells will be referred to as the "radiation-resistant class of cells" to distinguish it from those cells whose capacity to participate in PFC responses is completely inhibited by much lower doses of irradiation (6).

The following experiments were designed to determine the sedimentation profile of the radiation-resistant class of cells. Normal spleen cells were sedimented, and each fraction was irradiated with a dose of 1500 rads. All of the cells in fractions sedimenting from 1 to 7 mm per her were cultured. Fractions containing less than 2×10^7 cells were divided in half, whereas fractions containing more cells were divided into aliquots of 10^7 cells for each culture. These cells were mixed with SRBC and 2×10^6 normal spleen cells. This number of normal spleen cells is capable of giving rise to PFC responses only in the presence of a large number of heavily irradiated cells (see Materials and Methods). Thus, in these experiments, the production of a PFC response in cultures

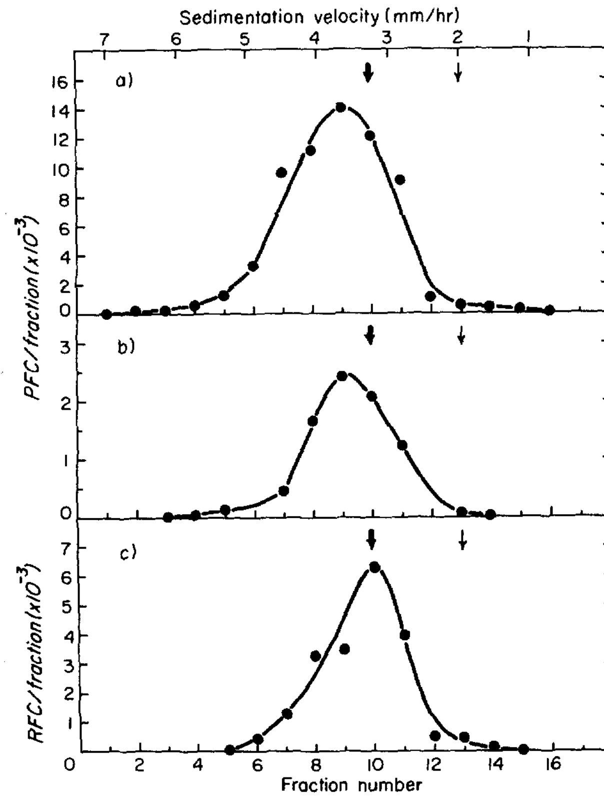

FIG. 2. *a*) Profile of the PFC response obtained when aliquots of normal spleen cells from each fraction are cultured with heavily irradiated spleen cells and SRBC. *b*) Profile of the PFC response obtained when 2×10^6 normal spleen cells are cultured with aliquots of heavily irradiated cells from each fraction and SRBC. *c*) Sedimentation profile of RFC in fractions of sedimented spleen cells. In each panel, the heavy and light arrows indicate the position of the peak number of nucleated cells and erythrocytes, respectively.

TABLE I

PFC Produced by Sedimented Cells as Compared to Unsedimented, Control Cells

Exp. No.	Number of PFC per 10^6 cells cultured	
	Obtained	Expected
1	92	74
2	155	150
3	150	148
4	125	131
5	149	133
6	271	211
7	150	99

set up from a given fraction indicates the presence of an adequate number of cells capable of facilitative activity in that fraction. Control cultures consisted of 10^7 irradiated, unsedimented cells mixed with 2×10^6 normal spleen cells and SRBC.

To obtain the sedimentation profile of the radiation-resistant class of cells, the total PFC response in each fraction was plotted on a graph. Fig. 2 *b* shows the results of a typical experiment. In four experiments, the largest numbers of PFC were produced in cultures containing irradiated spleen cells sedimenting at 3-4 mm per hr (peak, 3.6 ± 0.2 mm per hr).

The total number of PFC produced in cultures containing irradiated cells subjected to sedimentation was similar to the number produced by cultures containing an equal number of irradiated control cells. Thus, all of the expected facilitative activity was recovered in the fractions of sedimented cells.

The results of the experiments in this and the preceding section show that the sedimentation velocities of the radiation-resistant cells and the cells giving rise to PFC are similar. Therefore, the procedure of velocity sedimentation, used alone, failed to separate the various postulated classes of cells from each other. However, this problem was resolved by the experiments described in the following sections.

Combined Use of Velocity Sedimentation and Rosette Formation.—When normal, unimmunized spleen cells are briefly exposed to foreign erythrocytes a small proportion of the nucleated cells firmly bind erythrocytes to their surface to form a structure resembling a raspberry. This structure is known as a rosette (8, 9). Formation of rosettes with foreign erythrocytes appears to be highly specific, since cells forming rosettes with SRBC rarely form rosettes with CRBC and vice versa, when both of these kinds of erythrocytes are present. The nucleated cell in a rosette is referred to as a rosette-forming cell (RFC).

Since RFC are, in effect, altered in size by the binding of SRBC to their surfaces, it can be expected that rosettes would have a higher sedimentation velocity than cells not forming rosettes[1] (11). Thus, the procedure of rosette formation should be useful in separating RFC from the remainder of the spleen cell population.

Profiles of RFC and Rosettes.—The initial step was to determine the sedimentation profile of RFC in a normal spleen cell population. Normal spleen cells were sedimented, the fractions collected and mixed briefly with SRBC to allow rosette formation, and the number of RFC in each fraction was counted. In a series of five experiments, it was found that the peak number of RFC was recovered in fractions sedimenting at 3.2 ± 0.1 mm per hr (Fig. 2 *c*). Thus, the RFC profile lies entirely within the profiles of cells capable of giving rise to PFC and of cells having a facilitative function. This result is in keeping with

[1] J. C. Kennedy, unpublished data presented at FASEB meeting, Atlantic City, April, 1967.

the view that RFC may be one of the classes of cells that participates in PFC responses.

The next step was to determine whether or not RFC within rosettes could be separated from the remaining spleen cell population by velocity sedimentation. Spleen cells, containing a known number of preformed rosettes, were subjected to sedimentation. It was found that up to 80% of the rosettes could be recovered in fractions sedimenting at 8–16 mm per hr (Fig. 3).

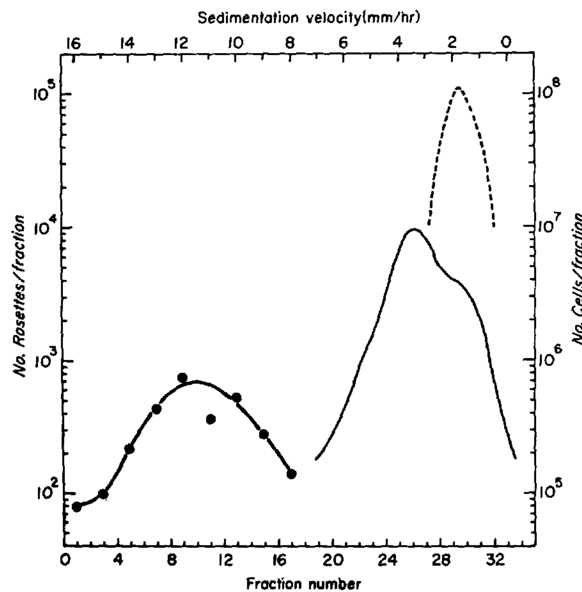

FIG. 3. Sedimentation profile of the rosettes (●——●) and the remaining spleen cells (——) and erythrocytes (- - - -) obtained by sedimenting spleen cells containing preformed rosettes.

Examination of the fractions containing the majority of rosettes showed that these fractions also contained clumps of nucleated cells which were of the same size as the rosettes. These clumps were composed of up to 10–15 cells. The ratio of clumps: rosettes was in the range of 5–10:1.

Results of Mixing Experiments.—The next experiments were designed to determine whether or not rosettes, or spleen cells depleted of rosettes, were capable of giving rise to PFC responses when cultured either alone or in the presence of heavily irradiated spleen cells.

Spleen cells containing preformed rosettes were sedimented, and fractions containing rosettes were collected and pooled. Varying numbers of rosettes (60–350), either alone or mixed with 10^7 heavily irradiated spleen cells, were cultured. Rosettes cultured alone always failed to give rise to PFC. When they

were cultured with irradiated spleen cells they gave rise to a small number of PFC (50 per culture) in only one of eight experiments.

When the spleen cells in fractions sedimenting from 1 to 7 mm per hr, known to be largely depleted of RFC, were cultured in the presence of heavily irradiated cells, these fractions still gave rise to some PFC. However, the number was always much less than the number of PFC produced by unsedimented, control cells (Table II). Thus, it may be concluded that the removal of RFC results in a significant loss of the capacity to produce PFC by the remaining spleen cells. Furthermore, RFC within rosettes are not capable of giving rise to significant numbers of PFC when cultured alone or in the presence of heavily irradiated spleen cells.

The results presented thus far can be interpreted in two ways. The lack of PFC production by rosettes, and decreased PFC production by spleen cells

TABLE II

PFC Produced by Sedimented Cells Largely Depleted of RFC as Compared to Unsedimented, Control Cells

Exp. No.	Number of PFC per 10^6 cells cultured	
	Sedimented cells largely depleted of RFC	Unsedimented, control cells
1	61	132
2	28	72
3	47	149
4	8	61
5	16	116

depleted of RFC suggests that a third class of cells, essential for maximum PFC production, may still be present in the spleen cell fractions largely depleted of RFC. An alternative interpretation is that the procedure of rosette formation and velocity sedimentation have, in themselves, damaged the cells subjected to these procedures so that they can no longer give rise to immune responses in culture. To test these possibilities, mixing experiments were done.

Normal spleen cells containing preformed rosettes were sedimented. Fractions containing rosettes were pooled and aliquots of this pool were added back to one half of each fraction of sedimented spleen cells (1–7 mm per hr) depleted of RFC. To each culture, 10^7 heavily irradiated spleen cells and 2×10^6 SRBC were also added. The results (Table III) show that the readdition of rosettes to fractions depleted of RFC significantly increased the PFC response. The peak PFC response, when rosettes were added back, was present in fractions sedimenting at 4 ± 0.2 mm per hr with a smaller peak at 3 mm per hr. However, the PFC response produced by fractions to which rosettes were not added back still peaked at 3.6 mm per hr. The profile of the PFC response in a typical

experiment is shown in Fig. 4. Thus, the possibility that the separation procedures damaged cells, rendering them incapable of generating PFC responses in culture, can be excluded. Rather, these results suggest that the readdition of rosettes to fractions of spleen cells largely depleted of RFC detects the presence of a third class of cells. Since this class of cells is not removed from the spleen cell population by the procedure of rosette formation, it can be concluded that these cells do not form rosettes.

As mentioned in the preceding section, the fractions containing rosettes also contained clumps of nucleated spleen cells. Therefore, the possibility that the clumps of spleen cells cosedimenting with rosettes were responsible for the

TABLE III

PFC Produced by Sedimented Cells Largely Depleted of RFC, either Cultured Alone or with Readded Rosettes

Exp. No.	PFC produced by rosettes alone	Number of PFC per 10^6 cultured cells produced by:		
		Sedimented cells alone	Sedimented cells plus rosettes	Control cells
1	0 (60)*	10	85 (50)*	75
2	0 (100)	70	235 (240)	233
3	0 (350)	75	250 (110)	120
4	0 (225)	24	80 (60)	64
5	0 (175)	20	117 (175)	146

* The figure in brackets is the number of rosettes either cultured alone or added back to each culture containing 10^7 irradiated cells and 10^6 cells from sedimented fractions largely depleted of RFC.

restoration of PFC activity in culture was investigated. Normal spleen cells, not containing preformed rosettes, were sedimented in the usual manner. Fractions containing clumps of spleen cells were collected and pooled. Aliquots of these fractions either were cultured with heavily irradiated spleen cells or were readded to one half of each fraction of spleen cells, sedimenting from 1 to 7 mm per hr and cultured with heavily irradiated spleen cells. Mixtures of clumps of nucleated spleen cells and heavily irradiated spleen cells did not give rise to PFC responses. Furthermore, the PFC response in fractions to which clumps of nucleated spleen cells were added was not enhanced, and the peak PFC response still was present in fractions sedimenting at 3.6 mm per hr. Thus, it is unlikely that the clumps of nucleated spleen cells cosedimenting with rosettes contain significant numbers of cells capable of participating in the immune response.

Effect of Rosette Formation on the Capacity of Heavily Irradiated Spleen Cells to Facilitate PFC Responses.—These experiments were designed to determine whether or not RFC are identical to the cells capable of functioning after a

large dose of radiation. Therefore, spleen cells containing preformed rosettes were sedimented; the fractions containing preformed rosettes were pooled and irradiated with a dose of 1500 rads. Similarly, each fraction of spleen cells (1–7 mm per hr) largely depleted of RFC was also irradiated. Either irradiated rosettes (up to 10^3 per culture) or irradiated cells from RFC-depleted fractions were mixed with 2×10^6 normal spleen cells, 2×10^6 SRBC, and cultured. It was found that irradiated rosettes failed to facilitate PFC production by the

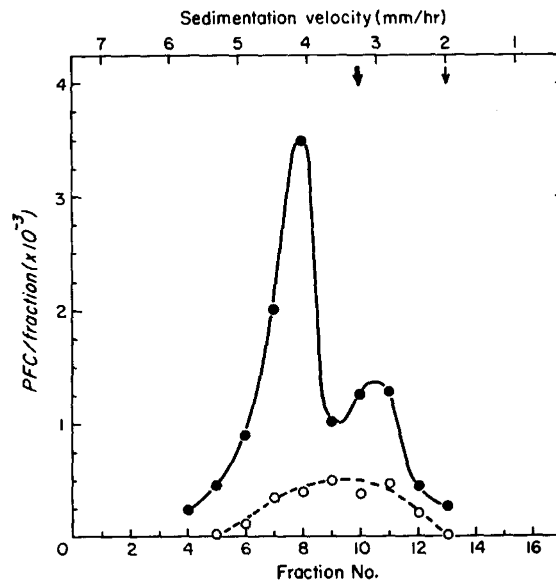

FIG. 4. Profile of the PFC response obtained when rosettes are added back to fractions of spleen cells largely depleted of RFC (●——●), as compared to the PFC response produced by fractions largely depleted of RFC to which rosettes were not readded (○- - - -○). Aliquots of each fraction were cultured in the presence of heavily irradiated spleen cells and SRBC. The heavy and light arrows indicate the position of the peak number of nucleated cells and erythrocytes, respectively.

normal cells. However, the heavily irradiated spleen cells depleted of rosettes still facilitated the production of large numbers of PFC. The total number of PFC produced in these experiments was similar to that found in the earlier experiments when sedimented spleen cells not containing preformed rosettes were irradiated and cultured with normal spleen cells. Furthermore, the profiles of the PFC response in both kinds of experiments were similar. Thus, it is unlikely that RFC are identical to the class of spleen cells capable of facilitating PFC formation after a large dose of radiation.

Specificity of PFC Responses to SRBC and CRBC.—The formation of rosettes

by nucleated spleen cells in the presence of two non-cross-reacting types of erythrocytes appears to be highly specific (8, 9). This knowledge was used in experiments designed to test whether or not the removal of RFC having specificity for SRBC had any effect on the capacity of spleen cells largely depleted of SRBC-RFC to respond to CRBC, and vice versa.

In two experiments rosettes were formed with SRBC and the spleen cell population containing preformed rosettes was sedimented. The fractions of spleen cells largely depleted of SRBC-RFC were cultured in the presence of either 2×10^6 SRBC or 2×10^6 CRBC. In two separate experiments rosettes were formed with CRBC, and the fractions largely depleted of CRBC-RFC were cultured with either SRBC or CRBC. The results (Table IV) show that the PFC response to the RBC with which rosettes were formed was diminished, while the PFC response to the other RBC was not affected. Thus, it can be

TABLE IV

PFC Response to SRBC and CRBC when Rosettes are Formed either with SRBC or CRBC

Exp No.	RBC used in rosette formation	Number of PFC per 10^6 cells produced to:			
		SRBC		CRBC	
		Control cells	Sedimented cells	Control cells	Sedimented cells
1	SRBC	109	26	104	151
2	SRBC	188	39	205	309
3	CRBC	95	200	300	120
4	CRBC	64	82	54	7

concluded that rosette formation with one type of RBC resulted in a significant decrease in the response to that RBC but not to the non-cross-reacting RBC.

The profile of the PFC response to the RBC not used in rosette formation showed that the peak response occurred in fractions sedimenting at 3.6 mm per hr. This result suggests that the cells giving rise to PFC responses to these types of erythrocytes are similar in size, but belong to separate subpopulations of spleen cells. This is in keeping with the results of limiting-dilution experiments suggesting that at least one of the classes of cells required for the generation of PFC is restricted in the capacity to respond to two non-cross-reacting antigens (5).

DISCUSSION

The development of tissue culture techniques for the generation of hemolysin-forming cell responses by mouse spleen cells has provided an opportunity to study the cellular events underlying these responses. On the basis of limiting dilution experiments, Mosier and Coppleson (1) have suggested that three cell classes are necessary for the generation of immune responses to SRBC by

mouse spleen cells in culture. They reasoned that one of the classes of cells has the capacity to adhere to plastic, and the other two classes are present in the nonadherent population. The adherent population of cells is still capable of facilitating PFC production by the nonadherent cells after exposure to 1000 rads (4). Similarly, we also have found a radiation-resistant class of spleen cells (D_{37} = 4.5 krads) capable of facilitating PFC responses by numbers of normal spleen cells too small to give rise to PFC by themselves (5, 6). Whether or not the radiation-resistant cells in these latter experiments are identical to those in the adherent population has not yet been determined. In contrast, the radiation survival of the small numbers of spleen cells giving rise to PFC has a D_0 of 100 rads (6).

Haskill et al., using density centrifugation as a means of separating cells, have also suggested that three classes of cells are required for the production of PFC in culture (3). One class can be recovered from each of the light, medium, and heavy density regions of the gradient. Cells from the light density region are capable of supporting PFC production in culture by cells from the heavy density region, even after the light density cells have been heavily irradiated.

In the experiments reported here, use was made of rosette formation (8, 9), velocity sedimentation (7), and irradiation to partially characterize the classes of immunologically reactive cells giving rise to immune responses in culture. The rationale for using these methods is that velocity sedimentation separates cells primarily according to their size. Rosette formation, in effect, increases the size of the cell which forms rosettes, thereby allowing separation of RFC from the cells not forming rosettes. Irradiation distinguishes between cells requiring the capacity for proliferation from cells not requiring this capacity during the immune response.

It was found that these methods detect three distinct classes of cells in the spleen. The peak number of cells capable of giving rise to PFC in the presence of a large number of heavily irradiated cells is present in fractions sedimenting at 3.6 mm per hr. Similarly, the peak number of the relatively radiation-resistant cells is also present in fractions sedimenting at 3.6 mm per hr. Thus, the use of velocity sedimentation alone does not separate these classes of cells. When RFC (peak sedimentation velocity, 3.2 mm per hr) are separated from the other spleen cells, the remaining cells lose a significant proportion of their usual capacity to give rise to PFC. However, the remaining cells, after irradiation, still retain the full capacity to facilitate PFC production by small numbers of normal spleen cells. In addition, irradiated RFC within rosettes do not facilitate PFC production by small numbers of normal spleen cells. Thus, it can be concluded that RFC are not identical with the radiation-resistant class of cells having a facilitative function.

The failure of RFC to give rise to PFC, either when cultured alone or with heavily irradiated cells, suggested that a third class of cells is also required for

the production of PFC in culture. It could be predicted that this class of cells, by definition, is not identical to the RFC or radiation-resistant class of cells. This prediction is borne out by the results of mixing experiments. When RFC within rosettes are added back to fractions largely depleted of RFC, and these mixtures are cultured in the presence of heavily irradiated cells, the fractions sedimenting at 4 mm per hr give rise to the largest number of PFC. A smaller peak of PFC was also produced by fractions sedimenting at 3 mm per hr. Since the peak PFC response occurred in fractions sedimenting at 4 mm per hr, this result suggests that these fractions contain a third class of cells. The smaller peak at 3 mm per hr can be explained by postulating that these fractions, not entirely depleted of RFC, were enriched for RFC by adding back rosettes. This enrichment, in the presence of a small number of the third class of cells and an excess number of the radiation-resistant class of cells, resulted in the production of PFC by these fractions.

In experiments making use of the specificity of rosette formation by RFC with SRBC and CRBC, it was found that rosette formation with one type of RBC significantly reduced the PFC response to that RBC by the spleen cells largely depleted of RFC, but did not affect the response of the same spleen cells to the RBC not used in rosette formation. The results of these experiments, taken with the results of the previous experiments, allow the conclusion that RFC are one of the classes of cells required for the production of PFC in culture. Furthermore, they are in keeping with the hypothesis that RFC are precursors of PFC.

In brief, the separation procedures used in these experiments resulted in a partial separation of three classes of immunologically reactive cells required for the production of PFC in culture. Some of the properties of the three classes of cells are presented in Table V.

It is of interest to compare the experiments reported here with those obtained in intact mice by Miller and Phillips.[2] Using the velocity sedimentation procedure, they found that the bone marrow contains RFC sedimenting at 3 mm per hr. When fractions of bone marrow are mixed with thymus cells and SRBC and injected into heavily irradiated mice, in a manner analogous to that described by Claman et al. (16), the largest number of PFC were found in those mice receiving bone marrow fractions sedimenting at 3 mm per hr. Thus, the marrow fractions containing RFC when mixed with thymus cells are active in giving rise to PFC in heavily irradiated mice. Furthermore, it is evident that RFC in bone marrow and the spleen have similar sedimentation velocities. This suggests that bone marrow RFC and spleen RFC may be identical. In separate

[2] R. G. Miller and R. A. Phillips. Sedimentation analysis of the cells in mice required to initiate an in vivo immune response to sheep erythrocytes. Manuscript submitted for publication.

experiments, Nossal et al. (17) have shown that the PFC found in the spleen of heavily irradiated mice receiving thymus-marrow cell mixtures are always derived from the marrow component. The results of the in vivo and in vitro experiments, taken together, support the hypothesis that the RFC is a precursor of PFC.

When fractions of thymus cells are mixed with bone marrow cells and SRBC and injected into heavily irradiated mice, the largest number of PFC is found in mice receiving thymus cells sedimenting at 3 mm per hr.[2] Thus, the sedimentation velocity of the immunologically reactive class of thymus-derived cells in vivo appears to be different than the sedimentation velocity of the third class of cells detected in the in vitro experiments reported here. This discrepancy raises the question of whether thymus cells are identical to the third class of spleen cells. It can be postulated that both kinds of cells do belong to the same

TABLE V

Some Properties of the Classes of Spleen Cells Required for the Generation of PFC in Culture

Property	Class of cells		
	1	2	3
Peak sedimentation velocity in mm/hr	3.2	3.6	4.0
Capacity to form rosettes	Present	Absent	Absent
Capacity for proliferation	Required	Not required	Required

class, but that the change in size reflects differing metabolic states. Further experiments are required to test this hypothesis.

SUMMARY

Three classes of immunologically reactive cells, differing only slightly in size from each other, are required for the production of hemolysin-forming cells in culture. The three classes of cells can be detected in the normal mouse spleen by the combined use of rosette formation, velocity sedimentation, and irradiation.

One class of cells (peak sedimentation velocity, 3.2 mm per hr) forms rosettes. The capacity of these cells to participate in immune responses to foreign erythrocytes is inhibited by relatively low doses of irradiation. These cells may be the immediate precursors of hemolysin-forming cells.

A second class of cells (peak sedimentation velocity, 3.6 mm per hr) facilitates the production of hemolysin-forming cells by small numbers of normal spleen cells. Their facilitative activity is resistant to a relatively large dose of radiation. They do not form rosettes.

The requirement of a third class of cells was deduced from the results of mixing experiments. Neither rosette-forming cells nor spleen cells largely

depleted of rosette-forming cells could give rise to hemolysin-forming cells when cultured either alone or in the presence of large numbers of heavily irradiated cells. However, when rosette-forming cells, cells depleted of rosette-forming cells, and heavily irradiated cells were mixed together, hemolysin-forming cells were produced. The peak responses were found in fractions sedimenting at 4 mm per hr. Thus, it is suggested that these fractions contain a third class of cells. This class of cells does not form rosettes, but its function is inhibited by relatively low doses of radiation.

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