IN VIVO REQUIREMENT FOR A RADIATION-RESISTANT CELL IN THE IMMUNE RESPONSE TO SHEEP ERYTHROCYTES*

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Two types of radiation-sensitive cells appear to be required to initiate an immune response to sheep red blood cells (SRBC)[1] in mice. One of these cells (the thymus-derived [T] cell) may be obtained from the thymus and the other (the bone marrow–derived [B] cell) from bone marrow, as was first demonstrated by the bone marrow–thymus synergism experiments of Claman et al. (1). In addition, in vitro studies of Roseman (2), Shortman et al. (3), Haskill et al. (4), and Osoba (5) indicate the requirement for a third, radiation-resistant cell to initiate an immune response in cell culture. Shortman et al. (3) refer to this third cell, which is resistant to gamma radiation up to doses of at least 1000 rads, as an accessory cell (A cell). An in vivo requirement for A cells cannot be satisfactorily demonstrated with the standard transplantation assays because they use irradiated recipients: any radiation-resistant cell required in the initiation will be supplied by these recipients and hence could even be absent from the transplanted cell suspensions without affecting the immune response produced by a test antigen. Until it is possible to demonstrate conclusively a requirement for A cells in vivo, the cell culture requirement for a radiation-resistant cell can be explained as a "feeder" effect related to the cultural conditions rather than an integral part of the initiation event (6).

Described below is an assay for a radiation-resistant cell required for the in vivo initiation of an immune response to SRBC. Using this assay, it has been possible to characterize some of the properties of this radiation-resistant cell and to show that it is similar to, and probably identical to, that radiation-resistant cell required in vitro. These experiments confirm that the radiation-resistant cells detected in vitro are an essential component of the antigen-sensitive unit.

Materials and Methods

Mice.—Male DBA/2J mice (purchased from the Jackson Laboratories, Bar Harbor, Maine) and C57BL/6JOci × C3H/HeOci mice (bred in our animal colony and hereafter

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[1] *Abbreviations used in this paper:* A, accessory; B, bone marrow-derived; BSA, bovine serum albumin; HRBC, horse red blood cells; HT, horse erythrocyte–treated; PBS, phosphate-buffered saline; PFC, plaque-forming cells; SRBC, sheep red blood cells; T, thymus derived.

referred to as C3B6F1 mice) were used in these experiments. Mice were kept three to a cage and allowed free access to food and water.

Irradiation.—All irradiated recipients were exposed to 950 rads of ionizing radiation using a ^{137}Cs irradiator at a dose rate of 102 rads/min. Unless specifically indicated, cells were injected into irradiated mice within 2 hr of irradiation. When cells were irradiated, the dose was 1300 rads, the cells being chilled in ice throughout irradiation.

Antigen.—SRBC and horse erythrocytes (HRBC) were obtained weekly from Woodland Farms Limited, Guelph, Ontario. Before use, either as antigen or as indicators in assays for antibody-producing cells, the erythrocytes were washed three times in phosphate-buffered saline (PBS).

Preparation of Cell Suspensions.—Cell suspensions were prepared by pressing tissue through a stainless steel screen as described previously (7). The cells were suspended either in PBS or in a special buffer (8) if they were to be separated on density gradients of Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden).

Culture System.—The method used was identical to that described by Marbrook (9) as modified by Osoba (10). The cells were cultured in Medium 1066 supplemented with 10% fetal calf serum and 20 μg/ml L-asparagine. We found that small changes in total cell density, even in cells incapable of contributing progenitor cells to the immune response, can alter the response appreciably and thus all cultures were made up to contain a total of 16×10^6 nucleated cells. Cultures were incubated with 2×10^6 SRBC or HRBC as antigen, and antibody-producing cells were determined 4 days later using the plaque-forming cell (PFC) assay of Jerne et al. (11).

In Vivo Measurements of Immune Response.—In some experiments, unirradiated mice were immunized and PFC were measured 4 days later. In other experiments, the cells to be assayed were injected into irradiated recipients (950 rads) together with antigen (10^8 erythrocytes/mouse) and PFC were measured in the spleens at the time of the maximum 19S PFC response as determined in separate kinetics experiments. For spleen cell transplantation, assays were performed on day 6; for mixtures of bone marrow and thymus, assays were carried out on day 8.

Cell Separation.—Density cell separation was performed using Ficoll density gradients at pH 5.5 as described previously (8). Modifications of this method involving the use of an A-XII zonal rotor to process large numbers of cells, and step gradients to obtain cells from discrete density intervals, have also been reported elsewhere (7).

Velocity sedimentation cell separation was also performed as described previously (12). A sterilizable glass sedimentation chamber (O. H. Johns Scientific, Toronto), 21.3 cm in diameter, was used. Cell loads were 9×10^8 cells in 100 ml of 0.3% bovine serum albumin (BSA) in PBS; a 0.6–2% BSA in PBS-buffered step gradient was used. Cells were sedimented for 4 hr at 4°C.

Adherent cell separations were done as originally described by Mosier (13). 1.5×10^8 nucleated spleen cells were placed in a large (150 mm) Petri dish in 15 ml of tissue culture medium (Medium 1066 supplemented with 10% fetal calf serum and 20 μg/ml L-asparagine) and incubated at 37°C for 1 hr. The supernatant was poured into new dishes and the incubation procedure was repeated. After the second incubation the cells still in the supernatant were centrifuged out of suspension (10 min at 250 *g* in sterile, siliconized 50-ml centrifuge tubes) and resuspended in tissue culture medium. This population of cells ("nonadherent spleen cells") is deficient in A cells. The dishes from the first incubation were flushed three times with 50 ml of PBS, refilled with tissue culture medium, and the cells stuck to the bottoms were scraped off with a rubber policeman and washed as above. This population of cells ("adherent spleen cells") is enriched in A cells.

RESULTS

A-Cell Deficiency in Mice 70 hr After Irradiation.—The results described in the introduction demonstrated the participation of a radiation-resistant cell in the initiation of an immune response in cell culture. These experiments showed that freshly irradiated suspensions of spleen cells are a good source of A cells. However, it was found that spleen cells removed from an irradiated mouse more than 24 hr after irradiation no longer have the ability to support the production of antibody-producing cells in vitro:[2] either A cells have left the spleen or they have become nonfunctional.

A prediction based on these observations is that the immune response produced by cell suspensions transplanted with antigen into irradiated recipients might be expected to vary depending on the time after irradiation at which the cells are transplanted and depending upon whether the transplanted suspensions contain A cells in addition to B cells and T cells. The results of experiments to test this prediction are shown in Table I. Spleen cells or mixtures of bone marrow and thymus cells were transplanted into freshly irradiated recipients or into mice that had been irradiated 70 hr before transplantation. When mixtures of bone marrow and thymus cells were tested, the immune response in 70-hr irradiated recipients was depressed compared to the response of the same number of cells in freshly irradiated recipients (A group, Table I). However, the response in mice that received spleen cells was the same regardless of the type of recipient used (B group, Table I). The tentative conclusion from this experiment is that suspensions of spleen cells, which are known to contain B, T, and A cells, function normally in either freshly or 70-hr irradiated recipients. However, mixtures of bone marrow and thymus cells seem to be deficient in A cells and lead to production of PFC in irradiated mice only when additional A cells are provided (C group, Table I).

A major problem in trying to use this assay routinely has been a lack of reproducibility. Preliminary work has indicated two factors that may contribute to this problem:

(*a*) Bone marrow contains appreciable numbers of A cells, up to 50% of the frequency found in spleen. Because this frequency is still quite low and because of the difficulty of obtaining large numbers of bone marrow cells for in vivo experiments, A-cell activity in bone marrow can only be reproducibly demonstrated using sensitive in vitro assays (see below). Although the number of A cells present in the bone marrow used in the above experiments was too low to give detectable activity, under some conditions there may be sufficient A cells to give a high "background" of activity.

(*b*) In vitro experiments have shown that irradiated spleen cells retain full

[2] El-Arini, M. O., and D. Osoba. Personal communication.

TABLE I

A-Cell Deficiency in Mice 70 Hr After Irradiation

Cells injected*	Normal recipients‡		Treated recipients§	
	No. survivors/ No. injected	PFC/spleen‖	No. survivors/ No. injected	PFC/spleen‖
Experiment 1				
A 5×10^6 bone marrow (BM) $+5 \times 10^7$ thymus	9/10	157[138–180]	6/10	29 [19–40]
B 2×10^7 normal spleen	10/10	532[417–676]	7/10	424[291–616]
C 5×10^6 BM $+5 \times 10^7$ thymus $+5 \times 10^7$ irrad. spleen¶	4/10	212[181–248]	6/10	183[127–268]
Experiment 2				
A 5×10^6 BM $+5 \times 10^7$ thymus	10/11	207[178–241]	12/17	25 [20–30]
B 1.5×10^7 normal spleen	10/10	1050[900–1220]	12/17	840[794–891]
C 5×10^6 BM $+5 \times 10^7$ thymus $+5 \times 10^7$ irrad. spleen¶	—	—	12/17	163[150–178]
Experiment 3				
A 5×10^6 BM $+5 \times 10^7$ thymus	—	—	8/10	24 [18–32]
Experiment 4				
A 5×10^6 BM $+5 \times 10^7$ thymus	—	—	5/8	18 [7–45]

* All recipients received 10^8 SRBC plus other cells as indicated; DBA mice were used in all cases.

‡ Mice injected within 2 hr of receiving 950 rads.

§ Mice injected 70 hr after receiving 950 rads.

‖ Geometric mean of PFC/spleen was assayed 8 days after transplantation; in these experiments spleens of mice receiving spleen cells were also assayed 8 days after transplantation. In all tables the values in brackets indicate one standard error on each side of the geometric mean.

¶ A spleen cell suspension was given 1300 rads within 2 hr of its injection. In experiment 1, control cell suspensions in which either bone marrow or thymus were omitted gave geometric means of 0 and 2, respectively, when assayed in treated mice.

A-cell activity after 3 days of incubation in vitro (see Table VII). In fact, A-cell activity even appears to increase under these conditions. Thus, the disappearance of A-cell activity from the spleens of irradiated mice cannot be attributed to the delayed effects of radiation on A cells; some other event, such

as migration of A cells from the spleens of irradiated mice, must be implicated. The observed radiation resistance and stability of A cells, together with the occurrence of A cells in bone marrow, may explain the lack of reproducibility

TABLE II

A-Cell Deficiency in Mice 24 Hr After Injection of 2 × 10^{10} Horse Erythrocytes

Cells injected*	Normal recipients‡			Treated recipients§		
	No. survivors/ No. injected	PFC/spleen‖		No. survivors No. injected	PFC/spleen‖	
Experiment 1						
A None	10/10	25,000	[22,000–29,000]	11/12	800	[600–1000]
B 8 × 10^7 irrad. spleen¶	11/11	29,000	[25,000–34,000]	9/12	5000	[4400–5600]
C 8 × 10^6 irrad. BM	11/11	25,000	[23,000–26,000]	12/12	780	[620–980]
D 8 × 10^7 irrad. thymus	11/11	21,000	[17,000–25,000]	12/12	1000	[840–1200]
Experiment 2						
A None	7/8	30,000	[27,000–33,000]	8/9	1680	[1400–2000]
B 7 × 10^7 irrad. spleen¶	7/8	35,000	[33,000–37,000]	8/8	14,000	[14,000–15,000]
C 1.2 × 10^7 irrad. BM	8/9	35,000	[33,000–38,000]	7/10	1400	[1200–1600]
D 7 × 10^7 irrad. thymus	8/8	32,200	[30,300–34,000]	10/10	1450	[1300–1600]
Experiment 3						
A None	11/12	39,000	[33,000–47,000]	9/9	6000	[5000–8000]
Experiment 4						
A None	11/12	33,000	[28,000–40,000]	9/10	830	[600–1200]
Experiment 5						
A None**	10/10	35,000	[30,000–41,000]	10/10	7700	[6300–9400]
Experiment 6						
A None**	9/10	43,000	[37,000–50,000]	10/10	5300	[4200–6000]
Experiment 7						
A None	5/5	24,000‡‡	[19,000–34,000]	5/5	310‡‡	[220–430]

* All recipients received 10^8 SRBC plus other cells as shown; DBA mice were used in all cases.
‡ Mice given no prior treatment.
§ Mice injected 24 hr after receiving 2 × 10^{10} horse erythrocytes intraperitoneally.
‖ Geometric mean of PFC/spleen assayed 4 days after injection of SRBC.
¶ Spleen cell suspension from normal mice given an irradiation dose of 1300 rads within 2 hr of its injection.
** As in § but only 5 × 10^9 horse erythrocytes were used.
‡‡ 7S PFC to SRBC assayed 7 days after SRBC injection.

in the assay using recipients 70 hr after irradiation. For these reasons an alternative in vivo assay for A cells was sought.

A-Cell Deficiency in Mice After Injection of Large Numbers of Horse Erythrocytes.—Many investigators have reported suppression of the immune response after injection of particulate matter such as colloidal iron or carbon particles

(14, 15). If this suppression is the result of inactivation of A cells, one might expect that the response could be restored by the addition of a radiation-resistant cell. To test this hypothesis, 2×10^{10} HRBC were injected into the peritoneal cavity of normal mice which were then returned to their cages for 24 hr; hereafter such mice will be referred to as horse erythrocyte–treated mice or HT mice. 1×10^8 SRBC were then injected into these mice and the PFC response specific for SRBC was measured 4 days later. The treated recipients showed up to a 40-fold reduction in the 19S PFC response compared to untreated controls (A groups, Table II). The response could be partially restored by injection of irradiated spleen cells (B groups) but not by injection of irradiated bone marrow or thymus cells (C and D groups). Using in vitro assays (see below) bone marrow was later shown to contain A cells, but their frequency is too low to be detected in this in vivo assay. Thus restoration occurred only when a cell population known to contain a significant number of A cells was added.

Additional experiments have shown that both irradiated peritoneal cells from normal mice and irradiated spleen cells taken from mice 7 days after they received a lethal dose of whole body irradiation followed by a transplant of 5×10^6 bone marrow cells could also give a partial restoration of the response in HT mice.[3] The response, however, has never been restored to more than 20% of that in untreated mice. The reason for this is not understood but may be related to the large number of irradiated cells needed to give even partial restoration and their probable toxic side effects.

To determine which cells are inactivated by the prior injection of HRBC, cells from various tissues were mixed with SRBC and spleen cells from HT mice, and the mixture was injected into 70-hr irradiated recipients. In addition, irradiated spleen cells from HT mice were tested for their ability to reconstitute bone marrow–thymus synergism in 70-hr irradiated recipients. The data from these experiments are shown in Table III. The following conclusions can be drawn:

(*a*) The immune defect in cells from HT mice can be restored only by the addition of large numbers of irradiated normal spleen cells; bone marrow or thymus cells fail to produce restoration (groups A–E).

(*b*) Freshly irradiated spleen cells from HT mice lack the function necessary to restore the response of bone marrow and thymus in 70-hr irradiated mice (groups F–I).

(*c*) No significant difference is observed when normal spleen cells or spleen cells from HT mice are transplanted into freshly irradiated recipients (groups J and K).

All of these observations are consistent with the hypothesis that the prime

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[3] Gorczynski, R. M. Unpublished data.

effect of treating mice with 2×10^{10} HRBC is to cause a decrease in the activity of radiation-resistant cells found in their spleens 24 hr later.

A Quantitative In Vivo Assay for Radiation-Resistant Cells.—The results

TABLE III

Immunocompetent Cells Found in Spleens of HT Mice

	Cells injected*	Irradiation of recipients	No. survivors/ No. injected	PFC/spleen‡
A	2×10^7 HT spleen	70 hr before transplantation	12/14	130 [90–190]
B	2×10^7 HT spleen $+10^7$ BM	70 hr before transplantation	12/17	207 [179–240]
C	2×10^7 HT spleen $+7 \times 10^7$ thymus	70 hr before transplantation	12/17	281 [227–350]
D	2×10^7 HT spleen $+5 \times 10^7$ irrad. normal spleen§	70 hr before transplantation	23/25	884 [790–1000
E	2×10^7 normal spleen	70 hr before transplantation	12/17	840 [790–890]
F	10^7 BM $+ 7 \times 10^7$ thymus	70 hr before transplantation	12/17	25 [19–30]
G	10^7 BM $+ 7 \times 10^7$ thymus $+ 5 \times 10^7$ irrad. HT spleen§	70 hr before transplantation	13/17	25 [19–32]
H	10^7 BM $+ 7 \times 10^7$ thymus $+ 5 \times 10^7$ irrad. normal spleen§	70 hr before transplantation	13/17	163 [150–180]
I	10^7 BM $+ 7 \times 10^7$ thymus	2 hr before transplantation	10/10	207 [178–241]
J	2×10^7 HT spleen	2 hr before transplantation	10/10	1050 [900–1220]
K	2×10^7 normal spleen	2 hr before transplantation	10/10	1010 [860–1200]

* All recipients received 4×10^8 SRBC and other cells as indicated; DBA mice were used in all cases.

‡ Geometric mean of 19S PFC/spleen against SRBC assayed 8 days after injection.

§ These spleen cell suspensions were irradiated within 2 hr of their injection. They were either from normal mice or mice treated 24 hr beforehand with 2×10^{10} HRBC.

presented in the previous section indicate that the best quantitative in vivo assay for A cells could be devised using HT mice, both as irradiated recipients and as donors of spleen cells. The tissue to be tested for A-cell content would be mixed with HT spleen cells (to provide B and T, but no A cells, i.e. a sus-

pension which is $B^+T^+A^-$) and transplanted into irradiated HT mice (which lack B, T, and A cells, i.e. are $B^-T^-A^-$).

Spleen cells from HT mice were injected into irradiated HT recipients (group A, Table IV). The immune response was severely depressed compared to the response of normal spleen cells transplanted into irradiated HT recipients (group B, Table IV). Further, the addition of large numbers of irradiated normal spleen cells ($B^-T^-A^+$) restored the PFC response to the value found for normal spleen cells transplanted into irradiated recipients (groups C and D, Table IV). Note that unirradiated HT spleen cells initiate a normal PFC response in freshly irradiated, untreated recipients (groups J and K, Table III). These data indicate that HT spleen cells have normal B- and T-cell activity, i.e. that they are $B^+T^+A^-$.

TABLE IV

The Basis for an In Vivo Transplantation Assay for Radiation Resistant Cells

	Cells injected*	Treatment of recipients‡	No. survivors/ No. injected		PFC/spleen§	
			Exp. 1	Exp. 2	Exp. 1	Exp. 2
A	1.5×10^7 HT spleen ($B^+T^+A^-$)‖	HT + Irr ($B^-T^-A^-$)	11/11	9/10	20 [14–28]	20 [8–52]
B	1.5×10^7 spleen ($B^+T^+A^+$)	HT + Irr ($B^-T^-A^-$)	8/8	7/10	196[160–241]	181[100–355
C	1.5×10^7 HT spleen‖ ($B^+T^+A^-$) + 5×10^7 irrad. spleen ($B^-T^-A^+$)	HT + Irr ($B^-T^-A^-$)	7/11	9/10	264[200–365]	240[170–340]
D	1.5×10^7 spleen ($B^+T^+A^+$)	Irr ($B^-T^-A^+$)	8/8	9/10	297[190–440]	380[260–560]

* All mice were given 10^9 SRBC and other cells as indicated; DBA mice were used in all cases.

‡ Recipient mice were given a radiation dose of 950 rads within 2 hr of use (Irr) or were given 5×10^9 HRBC intraperitoneally 24 hr before use and 950 rads within 2 hr of use (HT + Irr).

§ Geometric mean of PFC/spleen at day 6.

‖ HT spleen cells were from mice given 2×10^{10} HRBC 24 hr before use.

Fig. 1 shows how the above results can be used as a semi-quantitative assay for A cells. Irradiated HT recipients ($B^-T^-A^-$) received a constant number (2×10^7) of spleen cells from HT mice ($B^+T^+A^-$) and varying numbers of irradiated normal spleen cells ($B^-T^-A^+$). The PFC response was measured 6 days later. A linear relationship is observed when log (PFC/spleen) is plotted against the number of irradiated cells injected. The probable reasons for the exponential relationship between the PFC response and the number of A cells are not entirely clear but may be related to the in vivo interaction of the different cell types. However, regardless of the reason for these kinetics, the reproducibility of the assay indicates that these conditions can be used to estimate the activity of A cells in vivo.

Mosier (13) and Roseman (2) have shown that the radiation-resistant cells

required in vitro have the ability to adhere to plastic dishes. They separated suspensions of spleen cells into adherent and nonadherent populations and found that neither population alone was capable of initiating an immune response in vitro to erythrocyte antigens Addition of small numbers of adherent spleen cells restored the capacity of the nonadherent populations to undergo a normal immune response. Furthermore, the function of the adherent cells was not affected by exposure to large doses of ionizing radiation.

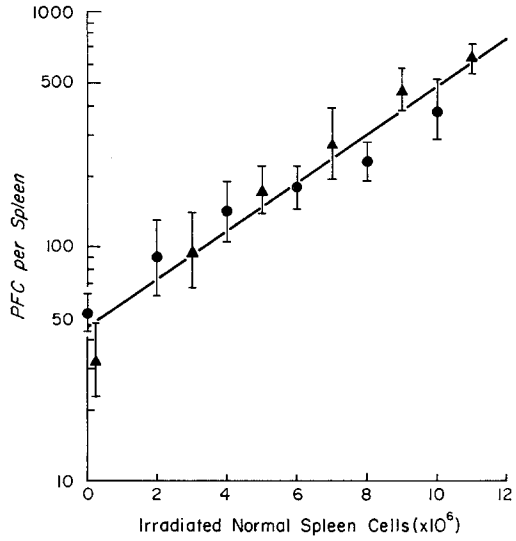

FIG. 1. In vivo assay for radiation-resistant cells. Irradiated HT mice as recipients were transplanted with 2×10^7 spleen cells from unirradiated HT mice together with 10^9 SRBC and a varying number of irradiated normal spleen cells. The PFC response to SRBC of the recipients was measured on day 6 and is recorded (with the standard error) in the figure above. Results of two experiments are shown as solid circles or solid triangles; each point represents a geometric mean for at least eight mice.

This same functional separation can be demonstrated in vivo, as shown in Table V. Here, the in vivo A-cell assay using irradiated HT mice as recipients shows that both nonadherent spleen cells and HT spleen cells (both presumably $B^+T^+A^-$) are inactive (groups A and B), but can be restored to activity by adding irradiated spleen cells ($B^-T^-A^+$) as is shown in groups C and D.

A Quantitative In Vitro Assay for Radiation-Resistant Cells.—In order to make a detailed comparison of the in vivo and in vitro radiation-resistant cell activity, it is necessary to establish a quantitative in vitro assay for radiation-resistant cells. Accordingly, 10^7 nonadherent cells obtained from the spleens of normal mice ($B^+T^+A^-$) were incubated with varying numbers of irradiated

cells from the spleens of normal mice ($B^-T^-A^+$). Since preliminary experiments had shown the importance of maintaining constant cell numbers in culture, varying numbers of irradiated nonadherent cells ($B^-T^-A^-$) were added to the cultures as necessary to maintain the total number of nucleated cells at 16×10^6. The number of PFC per culture was determined 4 days later. The data from this experiment are shown in Fig. 2 and Table VI. Also shown are the

TABLE V

A-Cell Activity of Nonadherent Spleen Cells In Vivo

Cells injected*	No. survivors/No. injected‡	PFC/spleen§
Experiment 1		
A 2×10^7 nonadherent spleen	9/10	25 [16–39]
B 2×10^7 HT spleen	7/10	26 [20–35]
C 2×10^7 nonadherent spleen $+7 \times 10^7$ irrad. normal spleen	9/10	154 [124–191]
D 2×10^7 HT spleen $+7 \times 10^7$ irrad. normal spleen	7/10	144 [116–178]
Experiment 2		
A 5×10^6 nonadherent cells	5/7	10 [8–13]
B 2×10^7 HT spleen	6/8	18 [12–27]
C 5×10^6 nonadherent cells $+6 \times 10^7$ irrad. normal spleen	4/8	74 [36–152]
D 2×10^7 HT spleen $+6 \times 10^7$ irrad. normal spleen	6/6	102 [72–144]

* All recipients received 10^9 SRBC plus other cells as shown at time of transplantation; DBA mice were used throughout.

‡ All recipient mice were given 5×10^9 HRBC 24 hr before transplantation and irradiated within 2 hr of injection of cells.

§ Geometric mean of 19S PFC/spleen 6 days after transplantation.

data obtained when irradiated HT spleen cells and irradiated adherent cells are assayed for their radiation-resistant cell content. For all populations tested, the relation between PFC response and cells tested is reasonably linear (unlike the in vivo assay, which gave an exponential relationship). To facilitate comparison, the data have been plotted logarithmically in Fig. 2 and all responses have been normalized to that of 2×10^6 irradiated spleen cells, taken as one. The lines through the points assume a linear response. Clearly, irradiated adherent cells are enriched about 2.5-fold and irradiated HT spleen cells are depressed at least 2-fold (probably more) in radiation-resistant cell content as compared to normal irradiated spleen cells.

Radiation Resistance of A Cells.—The data shown in Table I indicate that A-cell activity usually disappears from the spleens of irradiated mice within 3 days of irradiation. With the in vivo assay described above it is impossible to determine whether this loss of activity is the result of migration of A cells out of the spleen or the delayed effect of radiation on A cells. With the in vitro assay it is possible to separate these two factors since migration is not possible in culture. The experiment summarized in Table VII indicates that irradiated

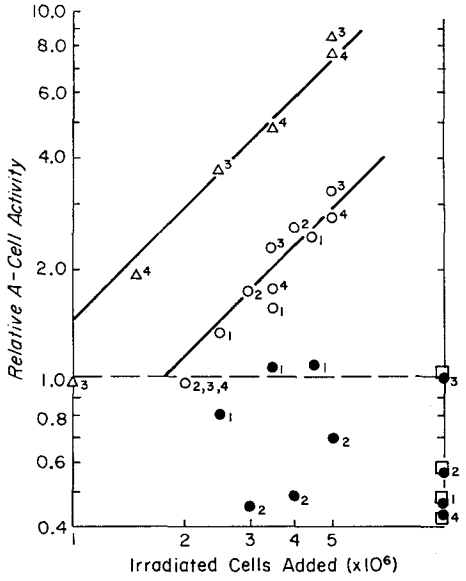

FIG. 2. In vitro assay for radiation-resistant cells. A description is in the text and Table VI. Different cell populations were assayed for A-cell content: open circles, normal spleen cells; open triangles, adherent spleen cells; closed circles, HT spleen cells; closed circle within square, nonadherent cells. The experiment number shown alongside the points on the curves enables the reader to refer to Table VI for the experimental data.

A cells are fully active 3 days after irradiation. Because freshly prepared nonadherent cells were required in the assay for A cells, these experiments had to have two sets of controls: one set to test the activity of the nonadherent cells at day 0 and another set to test the suspension prepared on day 3. Groups A and C show that all nonadherent populations were active when tested on freshly prepared, irradiated spleen cells. Groups B and D show residual A-cell contamination in the suspensions of nonadherent spleen cells. Groups E and F show that A-cell activity increases if spleen cells are incubated for 3 days before testing; group A gives the A-cell activity of the suspension before incuba-

TABLE VI

In Vitro Assay for a Radiation Resistant Cell

Cells cultured*	PFC/culture‡
Experiment 1	
6×10^6 irrad. nonadherent	100 [78–127]
2.5×10^6 irrad. normal spleen	290 [205–411]
3.5×10^6 irrad. normal spleen	337 [305–371]
4.5×10^6 irrad. normal spleen	527 [357–777]
3.5×10^6 irrad. HT spleen	171 [143–208]
4.5×10^6 irrad. HT spleen	232 [201–267]
5.5×10^6 irrad. HT spleen	238 [187–302]
Experiment 2	
6×10^6 irrad. nonadherent	69 [56–85]
2×10^6 irrad. normal spleen	122 [91–170]
3×10^6 irrad. normal spleen	215 [175–263]
4×10^6 irrad. normal spleen	319 [275–360]
3×10^6 irrad. HT spleen	56 [42–75]
4×10^6 irrad. HT spleen	60 [50–72]
5×10^6 irrad. HT spleen	85 [77–106]
Experiment 3	
6×10^6 irrad. nonadherent	190 [170–210]
2×10^6 irrad. normal spleen	184 [169–220]
3.5×10^6 irrad. normal spleen	426 [405–447]
5×10^6 irrad. normal spleen	602 [490–730]
1×10^6 irrad. adherent	180 [173–205]
2.5×10^6 irrad. adherent	690 [600–790]
5×10^6 irrad. adherent	1600 [1400–1780]
Experiment 4	
6×10^6 irrad. nonadherent	86 [78–95]
1.5×10^6 irrad. adherent	380 [346–419]
3×10^6 irrad. adherent	952 [840–1077]
5×10^6 irrad. adherent	1519 [1439–1602]
2×10^6 irrad. spleen	195 [187–202]
3.5×10^6 irrad. spleen	349 [315–387]
5×10^6 irrad. spleen	546 [491–607]

* In addition to the cells indicated, all cultures contained 10^7 nonadherent spleen cells, 10^6 SRBC, and varying numbers of irradiated nonadherent cells to maintain the cell density at 16×10^6/culture. DBA mice were used for all experiments.

‡ Geometric means of PFC per culture on day 4; four or five cultures were assayed for each group.

tion. One set of spleen cells (group E) were irradiated before incubation; this group is the equivalent of the in vivo assay, i.e., 3 days elapse between irradiation and testing. Comparison of the A-cell activity in groups A and E shows that, in contrast to the in vivo assay, preincubation of irradiated spleen cells

for 3 days in vitro enhances their activity. This enhancement is not dependent on irradiation since unirradiated suspensions (group F) also show enhanced activity resulting from preincubation. These data suggest that functional A cells probably exist in mice 3 days after irradiation, but that these cells either migrate out of the spleen to another organ or are prevented from functioning by some other mechanism. The reason for the increased A-cell activity following 3 days of preincubation is not clear from these experiments. Since the increase occurs even when the cells are irradiated before preincubation (group E), it is unlikely

TABLE VII

Survival of Irradiated A Cells In Vitro

Group; source of irradiated cells	PFC/culture*	
	Exp. No. 1	Exp. No. 2
Day 0 controls:		
A 10^7 spleen	93 [85–102]	179 [116–276]
B 10^7 nonadherent spleen	6 [6–7]	35 [23–53]
Day 3 controls:		
C 10^7 spleen	284 [207–390]	180 [114–285]
D 10^7 nonadherent spleen	32 [21–51]	26 [17–38]
Test of spleen cells cultured for 3 days:		
E 10^7 cultured cells‡ (irradiated on day 0)	949 [913–986]	1170 [1000–1300]
F 10^7 cultured cells‡ (irradiated on day 3)	1600 [1540–1660]	1680 [1430–1980]

* A-cell activity in the irradiated suspensions was determined by adding 10^7 nonadherent spleen cells ($B^+T^+A^-$) and 10^6 SRBC. In all groups PFC were measured 4 days after addition of the nonadherent spleen cells. The values shown are the geometric mean of at least three cultures.

‡ 2×10^7 normal spleen cells were placed in culture vessels without antigen. After 3 days the cells in these cultures were harvested, washed, and counted. The recovery of nucleated cells from these cultures were approximately 50%. 10^7 cultured cells were then mixed with 10^7 freshly prepared nonadherent spleen cells and placed in culture vessels to measure the recovery of A-cell activity in the cultured spleen cell suspensions.

that the increase in activity results from proliferation of A cells. It is possible that suspensions of spleen cells contain inhibitory cells or substances which disappear during the period of preincubation, but more experiments will be required to determine the precise mechanism for the enhancement in culture.

Comparison of Radiation-Resistant Cells In Vivo and In Vitro.—If both the in vivo and in vitro assays detect the same radiation-resistant cell, physical properties of the cell such as density or sedimentation velocity should be the same regardless of which assay is used for measurement. For the density comparison, suspensions of cells from the spleens of normal mice were separated on Ficoll density gradients, the various density fractions were collected, and

the cells were irradiated. For the in vitro assay 10^8 nucleated cells were separated in a 30 ml centrifuge tube and, after irradiation, added to cultures containing 10^7 unirradiated nonadherent spleen cells such that each culture received 10% of the cells from the fraction being assayed. The total number of cells in each culture was then made up to 16×10^6 by adding irradiated nonadherent cells as before. To obtain sufficient cells for the in vivo assay the cells were separated using a zonal centrifuge. Fractions were collected, irradiated, mixed with HT spleen cells, and injected into irradiated HT recipients such that each recipient

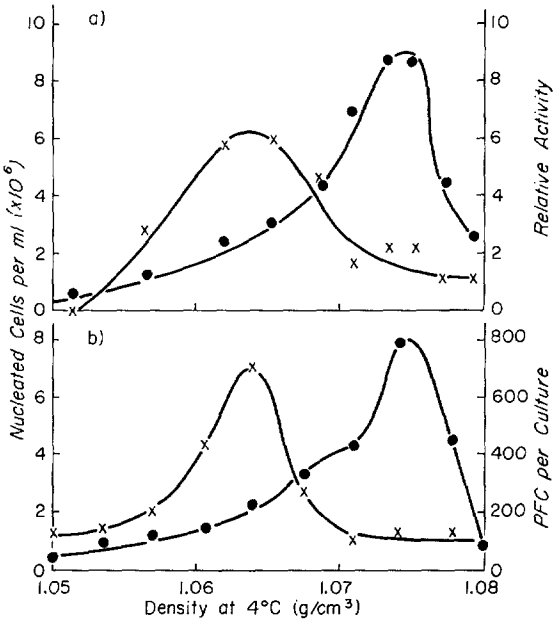

FIG. 3. Density profile of A cells. The upper panel gives the profiles of nucleated cells (solid circles) and A cells (crosses) using the in vivo assay. The lower panel shows the same data for the in vitro assay of A cells. For the in vivo assay at least eight mice were assayed for each point shown; for the in vitro assay at least four cultures were assayed.

received 5% of the cells from the fraction being assayed and 2×10^7 HT spleen cells. For both assays, in vivo and in vitro, SRBC were the antigen. The results (Fig. 3) show that the cells required in both assays have similar density profiles on a Ficoll gradient. For both, the modal density is 1.065 g/cm³. The width of the density distribution is also similar. Such data are consistent with the same cells being detected in each assay.

The sedimentation profile for radiation-resistant cells, as detected in vitro, was determined by separating suspensions of spleen cells according to their sedimentation velocity, irradiating the cells in the various fractions with 1300

rads, and adding these cells to 10^7 nonadherent cells in culture vessels. As before, irradiated nonadherent cells were added to the cultures as necessary to bring the total number of nucleated cells in each culture to 16×10^6. The data from this experiment (Fig. 4) clearly show that the radiation-resistant cells required in vitro comprise two populations of large cells with sedimentation velocities (s values) of 4.0 mm/hr and 5.3 mm/hr, respectively.

Because of the difficulties in separating large numbers of cells by velocity

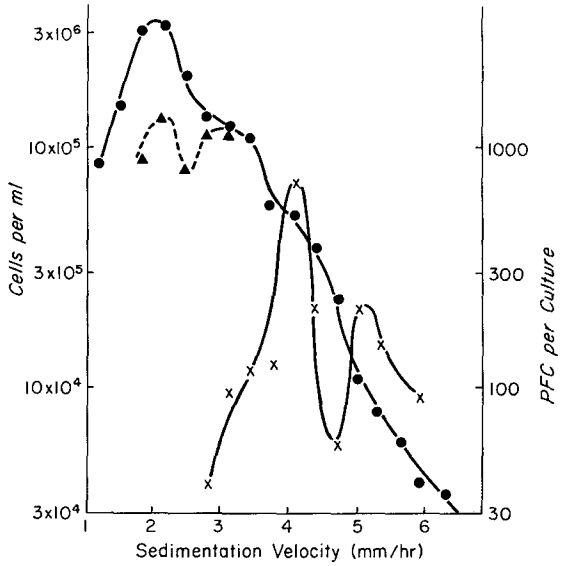

FIG. 4. Sedimentation profile of A cells. A suspension of spleen cells was separated by velocity sedimentation and individual fractions assayed for total cells (closed circles), nucleated cells (closed triangles), and A cells (crosses). A-cell activity was measured with the in vitro assay; a minimum of four cultures was assayed for each point.

sedimentation, a complete sedimentation profile for radiation-resistant cells using the in vivo assay was not obtained. Instead only two fractions were tested for in vivo activity. A suspension of spleen cells was separated by velocity sedimentation and divided into two pools: 2.5 mm/hr $< s \leq$ 3.5 mm/hr, and 3.5 $< s <$ 4.5 mm/hr. The former pool has already been shown to contain most of the B and T cells (16). Each pool was irradiated and tested by injection together with 2×10^7 spleen cells from HT mice into irradiated HT recipients. The data in Table VIII indicate that the radiation-resistant cell active in vivo is found in the pool of rapidly sedimenting cells, consistent with the observations made in vitro. Thus, both of the physical characterizations, size and density, indicate that the same cell is being detected in vivo and in vitro.

DISCUSSION

In this paper we have demonstrated a requirement for a radiation-resistant cell in the immune response of mice to SRBC both in vivo and in vitro and have developed quantitative assays for this cell under both conditions. The conclusion that the same cell is involved both in vivo and in vitro is greatly strengthened by the similarities of both the density and velocity sedimentation profiles under both assay conditions. Almost all previous data on the A cell

TABLE VIII

In Vivo Assay for Radiation Resistant Cells After Velocity Sedimentation

Cells injected*	No. survivors / No. mice injected‡		PFC/spleen§	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
2×10^7 normal spleen	10/10	10/10	3560 [2700–4650]	1920 [1580–2300]
2×10^7 HT spleen‖	12/12	12/12	323 [260–400]	300 [200–445]
2×10^7 HT spleen $+5 \times 10^7$ irrad. normal spleen (unfractionated)	9/12	11/11	3240 [2600–4000]	1740 [1360–2220]
2×10^7 HT spleen $+2.5 \times 10^7$ irrad. spleen (2.5–3.5 mm/hr)	10/11	9/10	305 [270–350]	530 [330–850]
2×10^7 HT spleen $+5 \times 10^6$ irrad. spleen (3.5–4.5 mm/hr)	10/11	10/10	1740 [1180–2580]	2400 [1820–3160]

* All recipients received 4×10^8 SRBC plus other cells as shown at time of transplantation; C3B6F1 mice were used throughout.

‡ All recipient mice were given 5×10^9 HRBC 24 hr before transplantation and 950 rads within 2 hr of transplantation.

§ Geometric mean of PFC/spleen 6 days after transplantation.

‖ Spleen cell suspension from mice treated with 2×10^{10} HRBC 24 hr before.

have come from in vitro experiments, and there have been suggestions (see, for example, Dutton et al. [6]) that it may only serve a "feeder" role. The demonstration that a cell with apparently identical properties is also required in vivo would seem to remove this possibility.

The identity of A-cell activity in vivo and in vitro could only be demonstrated after careful attention to the design of the in vitro assay. A major problem in any cell culture experiment is the separation of specific effects from nonspecific, feeder effects. A quantitative in vitro assay could be obtained only if both the total numbers of functional B and T cells, and the total number of nucleated cells, were maintained constant in all cultures. The resulting assay varies linearly with the number of A cells tested and appears to be independent of feeder effects (see Fig. 2). Using this assay, it is possible to determine the

relative frequency of A cells from various sources. Table IX summarizes our findings. Surprisingly, bone marrow is relatively rich in A cells. This observation does not necessarily invalidate the assumption that bone marrow is $B^+T^-A^-$ in transplantation experiments: A-cell activity has already diluted out for the actual cell numbers used. A-cell activity in bone marrow can only be detected in the more sensitive in vitro assay.

Other investigators have recognized the possibility of the feeder problem, although they made no attempt to distinguish between specific and nonspecific, feeder-like effects. Haskill et al. (4) suggested that perhaps the thymus-like cell was providing a nutrient and acting like a feeder layer; in their system the thymus-like effect was mediated across a dialysis membrane. Dutton et al. (6) observed that the supernatant from a suspension of adherent spleen cells cultured alone for 24 hr could cause an increase in the response of nonadherent

TABLE IX

Relative Frequency of A Cells from Various Sources

Source	Frequency*
Adherent spleen	$\geq 2.1/2$
Spleen	1
Bone marrow	1/2
HT spleen	1/6
Nonadherent spleen	1/15

* Normalized to a frequency of one for spleen. The numbers are rough estimates only.

spleen cells cultured with antigen. They suggest that some kind of "factor" is released by adherent cells, a factor which may play a specific immunological role or may merely be a nutrient factor. We have repeated this observation using irradiated spleen cells preincubated for 24 hr with antigen (SRBC) and found that the culture medium was then able to cause a large increase in the specific response of nonadherent spleen cells to both SRBC and HRBC.[4] The lack of specificity with this supernatant factor implicates a feeder effect. Although suspensions of nonadherent spleen cells may be as much as 20-fold depleted in A-cell activity, even the most carefully prepared suspensions cannot be assumed to be free of A cells; nonadherent cells cultured alone always give detectable, albeit low, responses. It is possible that the factors obtained from conditioned medium increase the effectiveness of the residual A cells in the nonadherent suspension. A similar effect may account for the increase in A-cell activity observed following incubation in culture (Table VII).

Our in vivo assay gives an exponential relationship between A-cell frequency

[4] Gorczynski, R. M. Unpublished data.

and response obtained. The reason for this deviation from the anticipated linearity may be merely indicative of the differences between cell cultures and irradiated mice as systems in which to study three-cell interactions, e.g. differences in the cellular interaction probability, or the possibility that some part of T-cell function may not be blocked by irradiation (17). However, the conclusions we have drawn from the comparison of in vitro and in vivo data rely only on the demonstration that A-cell activity can be measured quantitatively, and that the assays do not depend on feeder effects. Both assays meet this requirement.

Our in vitro density separation data compare rather closely with the data of Shortman et al. (3) and Haskill et al. (4), both of whom find that accessory cells are low-density, radiation-resistant cells. There are slight differences in the density profiles and in the absolute densities of the peaks, but these differences might be because of the fact that different strains of mice and different gradients were used in our experiments compared to theirs. However, there is a discrepancy between our data and those of Osoba (5). Using velocity sedimentation to characterize the various cells required in the initiation of an in vitro immune response to SRBC, he observed that the radiation-resistant component had an s value of 3.6 mm/hr. This value is considerably lower than the s values that we measured: 4.0 and 5.3 mm/hr. There are several factors that may account for this apparent discrepancy. First, Osoba used CBA mice while we worked entirely with DBA mice. Shortman et al. (3) have observed that different strains of mice showed different spleen cell density profiles so it is a possibility that s values may show strain differences. Second, the assay carried out by Osoba was different from the one used in the above studies. Some of the apparent differences may simply reflect differences in the assay for A cells.

The precise role that A cells play in the initiation of an immune response to SRBC is not clear. Shortman et al. (3) suggested that A cells provide a phagocytic function. As evidence for this suggestion, they show that a soluble antigen, polymerized flagellin, does not require A cells to initiate an antibody response in culture. In addition, the A-cell requirement for initiating a response to SRBC can be eliminated if sonicated SRBC are used as antigen. However, both Haskill et al. (4) and Shortman et al. (3) find that the density distribution of accessory cells is significantly different from that of the distribution of phagocytic cells. In experiments with peritoneal cells we have observed that incubation of these cells with SRBC for 4 hr before injection into irradiated HT mice resulted in a much larger immune response than injection of the same number of cells without preincubation with antigen. These data indicate that A cells act early in the initiation events, possibly by phagocytosis, but their precise role is not elucidated by such experiments. Mosier (18) has observed that cell-cluster formation is an early prerequisite step for the development of PFC in vitro. Perhaps the A cell is involved in bringing together the B and T cells; a similar sug-

gestion has been made by Talmage et al. (19). The experiments of Unanue and Cerottini (20) indicate that antigen is highly immunogenic when it becomes bound to the surface of macrophages. Such a situation might be highly effective in bringing together B and T cells and initiating their differentiation.

Our protocol for depleting mice of A cells by injecting large numbers of HRBC is reminiscent of protocols used to study reticuloendothelial system blockade (14, 15) and antigen competition (21). It could well be that these phenomena have a similar origin although the conditions used to study them are not directly comparable to those used here.

SUMMARY

Experiments have been done to establish whether the radiation-resistant or A cell has a specific function in the initiation of an immune response in mice to sheep erythrocytes (SRBC). All previous demonstrations using accessory (A) cells have involved in vitro assays and are possibly explainable as tissue culture artifacts. If A cells are essential, it should be possible to demonstrate their requirement in vivo. Therefore we first established such conditions. Two methods were found for creating an A-cell deficiency in vivo: (*a*) A cells disappear gradually from the spleens of irradiated mice, presumably by migration since A-cell function was shown not to be decreased by irradiation. If 3 days elapse between irradiation and transplantation of mixtures of bone marrow and thymus cells (which provide B and T but few A cells), the usual synergistic response does not occur. Addition of large numbers of freshly irradiated spleen cells to the mixture of bone marrow and thymus completely restores the immune response. (*b*) Injection of 10^{10} horse erythrocytes into mice suppresses A-cell activity in these mice 24 hr later; a much reduced response to SRBC is obtained when they are given at this time. The response can be partially restored if irradiated spleen cells are given with the SRBC. This observation formed the basis for a quantitative in vivo assay for A cells in which the magnitude of restoration by various suspensions of irradiated cells was used to estimate the A-cell activity of that suspension. A quantitative in vitro assay for A cells was also developed. It was essential for this assay that the total cell number, B-cell number, and T-cell number be kept constant and that only the number of A cells be allowed to vary. Only under these conditions was the response a linear function of the number of A cells added.

If the in vivo and in vitro assays are detecting the same class of radiation-resistant cells, the physical properties of the cells active in each assay should be identical. Spleen cells were separated on the basis of both density and sedimentation velocity. Fractions from both separation methods were tested for their content of A cells using both the in vivo and in vitro assays. The density and sedimentation profiles of A cells were similar in both assays.

The demonstration that a radiation-resistant cell is required in vivo and that

this cell has properties identical to the radiation-resistant cell required in vitro indicates that this cell (the A cell) is directly involved in the initiation of an immune response to erythrocyte antigens.

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