

IN VITRO AND IN VIVO STUDIES OF THE IMMUNE RESPONSE
TO SHEEP ERYTHROCYTES USING PARTIALLY PURIFIED
CELL PREPARATIONS*

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Several reports have described the initiation of a primary immune response in tissue culture (1-4). The technique of Mishell and Dutton (5), however, is of particular interest, as it utilizes a single cell suspension as the donor cell source, and this makes possible a detailed investigation of whether different cell types are involved in the in vitro immune response. Such investigations depend on the preparation of purified cell populations, or populations specifically depleted in a particular cell type.

The present paper describes the use of bovine serum albumin density gradients (6) to obtain partially purified mouse spleen cell preparations. The activity of these cells was tested by culturing them both in vitro, using the technique of Marbrook (7) based on that of Mishell and Dutton, and in vivo, using syngeneic irradiated mice as recipients (8-10). Sheep erythrocytes were used as antigen. The results have led to a partial characterization of three cell types involved in the in vitro response and have also demonstrated differences between the in vitro and in vivo assays of immune competence.

Materials and Methods

Animals.—Male and female mice, 7-9 wk old, of the highly inbred strains CBA (originally obtained from Harwell, Didcot, Berkshire, England) and C57BL (originally obtained from Dr. R. Bradley of the University of Melbourne, from Dr. L. W. Law of the National Institutes of Health, Bethesda, Md.) were used. (CBA × C57BL) F₁ mice were also used.

Antigen.—Sheep erythrocytes (SRBC)¹ were collected aseptically in Alsever's solution,

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¹ *Abbreviations used in this article:* BSA, bovine serum albumin; DDW, deionized distilled water; FCS, fetal calf serum; HVL, half value layer; MEM, Eagle's Minimal Essential Medium; PFC, antibody-forming cells; SRBC, sheep erythrocytes.

stored at least 7 days, and washed three times in a large volume of balanced salt solution before use. Routinely, 2×10^6 SRBC in culture medium were added to each culture. All sheep erythrocytes were obtained from a single sheep.

Cell Culture Medium.—Medium 199 and fetal calf serum (FCS) and some ingredients for Eagle's Minimal Essential Medium with calcium (MEM) (11), namely sodium bicarbonate, glutamine, amino acids A and B, vitamins, and phenol red, were obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. The concentrations of sodium chloride and sodium bicarbonate were modified from Eagle's original recommendations for use with a 10% CO₂ gas mixture. A modified MEM salt solution was prepared in 30 ml samples and stored (-16°C) until required to prepare 1 liter of medium. Final concentration of sodium chloride was 5.23 g/liter and sodium bicarbonate, 4.48 g/liter. The medium was also supplemented with sodium pyruvate (0.11 g/liter) and serine (0.21 g/liter), penicillin (4×10^4 units/liter). The medium was made up to 1 liter with deionized distilled water (DDW). Fetal calf serum (100 ml) was then added. The medium was sterilized by Millipore filtration (0.45μ) and stored at 4°C until required. Additional glutamine was added if the medium was not used within 5 days of preparation.

Preparation of Cells.—Tissues were removed aseptically from mice killed by cervical dislocation and bled from the jugular vein.

Single cell suspensions were prepared by teasing the tissue gently on a stainless steel sieve (80 mesh, 0.005 inch diameter wire) in a Petri dish containing 10 ml medium 199. One end of the spleen capsule was cut and the cells released by gentle stroking with bent forceps. Cells were separated and suspended in the medium with a siliconized Pasteur pipette. Clumped cells were allowed to settle in the Petri dish for 2–3 min, and the supernatant, which was virtually a suspension of single cells, was removed. This step is essential to obtain cell preparations suitable for density gradient analysis.

Cells were centrifuged (1100 rpm, 300 g, 7 min) and resuspended in BSA for gradient analysis. The supernatant was discarded.

Bone marrow cells were expressed from femurs by means of a syringe and attached needle containing cold medium 199. The marrow plugs were gently disrupted by aspiration through a 24 gauge needle. The suspension of cells was washed once, resuspended in cell culture fluid, and counted in a hemocytometer chamber.

Thymus lobes were removed, care being taken to avoid fascial tissue which might have contained mediastinal lymph nodes.

Density Gradient Analysis.—Bovine serum albumin (BSA) gradients were prepared as described previously by Shortman (6). The BSA was sterilized by two passages through 0.45μ Millipore filters. Spleen cell fractions of different density were pooled as desired, washed twice in the culture medium, and samples diluted in balanced salt solution before being counted on a Model B Coulter Counter equipped with a 100μ aperture. The lower threshold was set to eliminate erythrocytes from the count.

The optimal ratio of cells of different density in the final cell culture was determined by preliminary experiments in some cases, but was generally a matter of necessity, determined by the number of cells recovered from the gradient.

Cell Cultures.—The cell culture method used was similar to that described by Marbrook (7). The cells were cultured on dialysis membranes in 1 ml medium, containing 2×10^6 SRBC and inserted into a 9 ml reservoir of medium. In some experiments two cell populations were cultured in the same vessel, separated by a nucleopore membrane (0.8μ pore size).

Cultures were placed in a humidified incubator, 37°C with a constant flow of gas comprising 10% CO₂, 7% O₂, and 83% N₂.

Cultures were harvested at 3 days, and antibody-forming cells (PFC) to SRBC were assayed using the Cunningham and Szenberg (12) modification of the Jerne plaque technique (13) using guinea pig complement. Results reported are the mean of three cultures. Calcula-

tions to obtain density-distribution profiles were as follows: $Y = (\text{PFC per } 10^6 \text{ cells added to culture}) \times (\text{number of cells per fraction}) / (\text{density increment of that fraction})$, where Y was finally expressed as a percentage of the maximum response in that particular experiment. $X =$ density of each fraction.

In Vivo Assay of Spleen Cell Responsiveness to SRBC.—Spleen cells were injected intravenously into 7–9 wk old syngeneic mice which had been X-irradiated previously on the same day with 800 rads. Antigen (6×10^8 SRBC) was injected simultaneously (8–10).

Animals were killed 7 days later, their spleens were teased, and a sample was assayed for PFC using the Cunningham and Szenberg assay system.

Calculations to obtain density profiles were as follows: $Y = (\text{PFC per spleen}) \times (\text{number of cells per fraction}) / (\text{density increment})$ per (number of cells injected), where Y was finally expressed as a percentage of the maximum response in that particular experiment.

Phagocytic Cells.—Phagocytic cells were assayed by culturing irradiated cell fractions under the usual conditions, for 4 hr only, and then harvesting and preparing duplicate cell smears. Slides were stained with Giemsa stain and $8\text{--}10 \times 10^3$ cells scanned per slide, and the proportion of cells which had engulfed one or more SRBC's was estimated.

Preparation of Anti-H2 Sera and Incubation of PFC.—The antisera were very kindly prepared by Dr. Graham Mitchell according to the method described by Miller and Mitchell (14). The anti-CBA serum was prepared in C57BL mice, and the anti-C57BL serum in CBA mice. Normal CBA serum was used as a control. Cells to be assayed (in 0.1 ml) were treated with $15 \mu\text{l}$ antiserum 4°C , 30 min before assay. Guinea pig complement and SRBC were then added, and the assay was carried out as usual.

Irradiation Procedures.—Mice were given a whole body dose of 800 rad under maximum backscatter conditions, using a Philips RT 250 machine operating at 250 kv, HVL (half value layer) was 0.8 mm. Cu and dose rate 127 rad/min. Mice were irradiated in groups of 10 in plastic containers flushed with a constant flow of air. Opposing fields were used.

Spleen cell fractions were X-irradiated in Falcon plastic tubes (No. 2001, 17×100 mm) at a focal distance of 50 cm, with a dose rate of 746 rad/min. The cells were suspended in ice-cold culture medium which completely filled the tube.

RESULTS

The results are presented in two sections. The first section describes studies comparing in vitro and in vivo assays of the immune competence of partially purified spleen cell populations. In the second section, evidence is presented for the presence of a three cell interaction in the in vitro response to SRBC.

Comparison of In Vivo and In Vitro Responses of Spleen Cell Fractions to SRBC.—

A direct comparison of activity of fractions prepared using a BSA density gradient: Mouse spleen cells (C57BL \times CBA) F_1 , were teased and fractionated. Fractions were washed, counted, and their activity assayed in vivo and in vitro, using equal numbers of cells from each fraction. Results are shown in Fig. 1. (Similar results were obtained using CBA mice as donors, see Fig. 6). For ease of discussion, regions of the gradient have been designated fractions 1–4. Fraction 1 consists of cells in the density range less than 1.0600 g/cm^3 $1.0600 <$ fraction 2 > 1.0645 ; $1.067 <$ fraction 3 > 1.0695 ; $1.0700 <$ fraction 4 $> 1.075 \text{ g/cm}^3$.

Fig. 1 indicates that there was a difference in the in vivo and in vitro profiles of responsiveness to SRBC. Fraction 3, the peak of the in vivo activity, functioned very poorly in vitro. Fraction 2 was the peak of in vitro activity and corresponded to a peak of in vivo activity.

Confirmation of the profiles illustrated by Fig. 1 is shown in Fig. 2, which shows the dose response curves of fractions 2 and 3 in vivo and in vitro. It is evident that, in vivo, fraction 3 was more active than fraction 2 at all cell

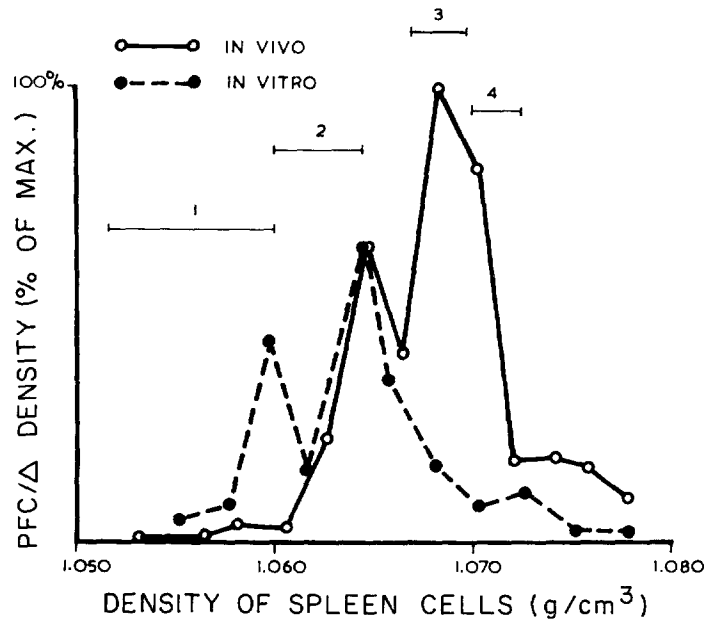


FIG. 1. Comparison of in vivo and in vitro responsiveness to SRBC of spleen cell fractions from normal (CBA × C57BL) F₁ mice. Cells in different regions of the gradient have been designated fractions 1-4 for convenience.

concentrations. On the other hand, fraction 3 had very low activity in vitro at all cell concentrations and fraction 2 showed much higher activity.

Role of the irradiated host in the in vivo assay: The difference in the activity of fraction 3 spleen cells in vivo and in vitro suggested that the irradiated animal in the in vivo assay was in fact contributing to the adoptive immune response. Macrophages and/or other cells from the irradiated recipient have been implicated in the adoptive immune response (4, 15, 16) and it was thought that the lack of in vitro activity of fraction 3 may have been due to a depletion of macrophages in this fraction. Since preliminary investigations indicated that the light density regions of the gradient were rich in macrophages, an experiment was carried out in which cells from the light density fraction 1 were

added to cell fractions from other regions of the gradient. The profile thus obtained was compared with the profile of fractions without added cells. The results are shown in Fig. 3.

It is evident that the in vitro profile obtained by adding fraction 1 cells to cells from other regions was now more similar to the in vivo profile shown in

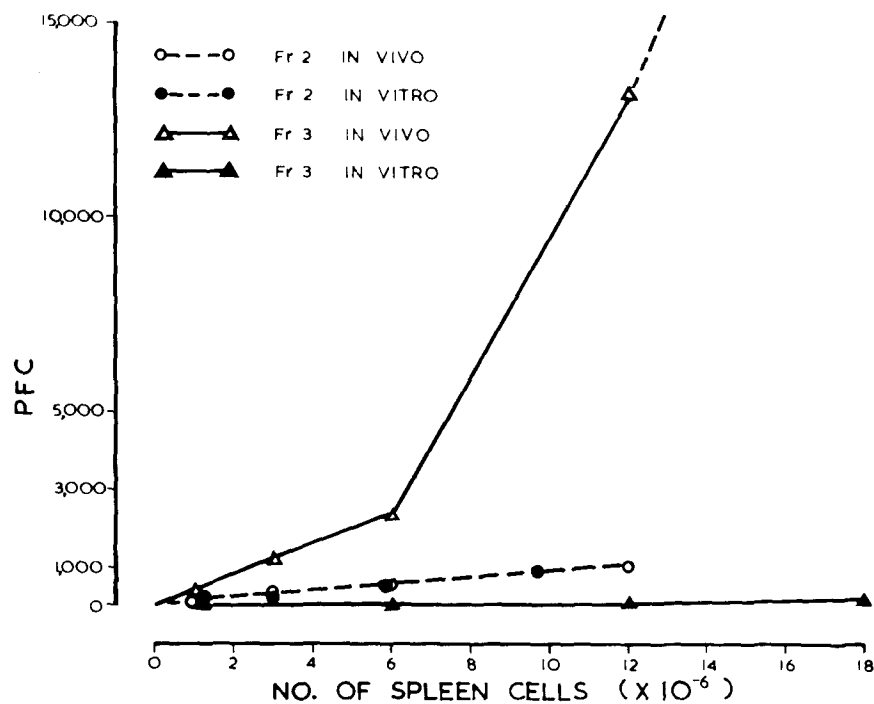


FIG. 2. Dose response curves of fractions of normal spleen cells (F_1) assayed in vivo and in vitro using SRBC as antigen. The fractions are defined in Fig. 1. The ordinate scale represents the number of PFC/spleen (in vivo) or the number of PFC/culture (in vitro).

Fig. 1. Fraction 2 was stimulated by approximately 60% on addition of fraction 1, while fraction 3 was stimulated 20-30-fold in this experiment. A similar effect was obtained if cells in fraction 1 were irradiated (1000 rad) before addition to fraction 3. Typical results are shown in Table I. These data are consistent with the idea that fraction 1 contained a radiation-resistant component which stimulated cells in fraction 3 to yield a greater immune response.

Density profile of the radiation-resistant stimulatory cell: A density profile of the radiation-resistant stimulatory cell was obtained by adding to cells from fraction 3 cells from other density regions which had previously been irradiated (1000 rad). Results are shown in Fig. 4. This figure also shows a density profile

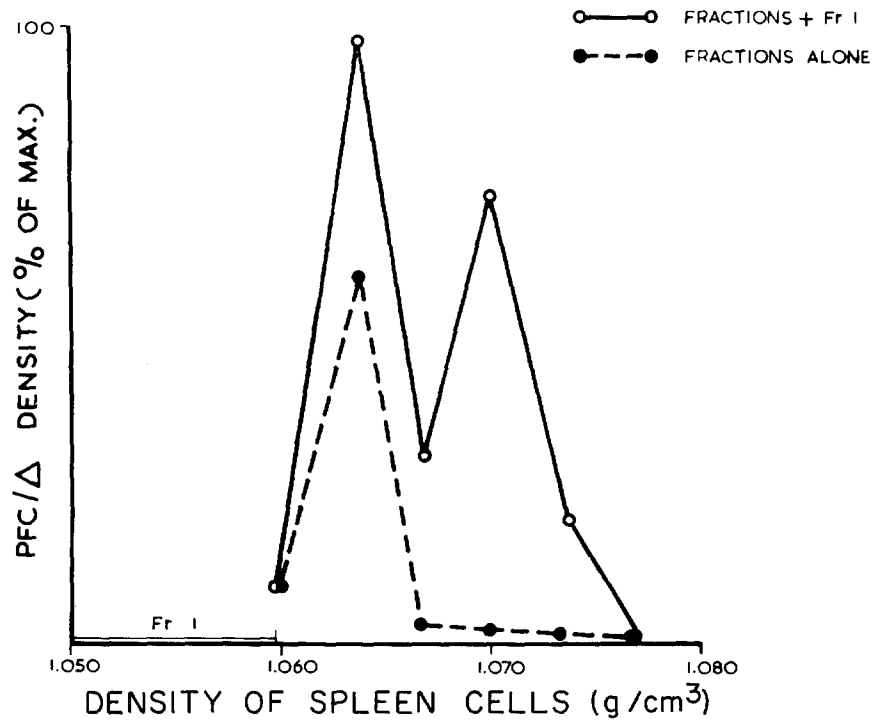


FIG. 3. Effect of fraction 1 on the response of other normal spleen cell fractions to SRBC in vitro. Fractions alone contained 10×10^6 cells per culture. The effect of fraction 1 was shown by adding 5×10^6 fraction 1 cells to 10×10^6 cells of the other fractions.

TABLE I
Effect of X-Irradiation on Activity of Spleen Fraction 1

	Spleen cell source*	Number of cells/ culture ($\times 10^{-6}$)	PFC/culture
Exp. 1	Fr 1‡	5	12
	Fr 1 (1000 rad)	5	<2
	Fr 3	8	30
	Fr 3 + Fr 1	8 + 5	214
	Fr 3 + Fr 1 (1000 rad)	8 + 5	140
Exp. 2	Fr 1	5	270
	Fr 1 (1000 rad)	5	15
	Fr 3	5	60
	Fr 3 + Fr 1	5 + 5	1130
	Fr 3 + Fr 1 (1000 rad)	5 + 5	226

* Normal CBA spleen cells were fractionated on a BSA density gradient. Fractions 1 and 3 (defined in Fig. 1) were prepared and cultured in vitro. Where indicated, Fraction 1 was irradiated (1000 rad) in vitro, before culture.

‡ Fr, fraction.

of the phagocytic cells in the same density fractions. An in vitro response profile is shown for comparison.

It is evident that the radiation-resistant cell profile did not correlate well with the profile of phagocytic cells. The main peak of the radiation-resistant

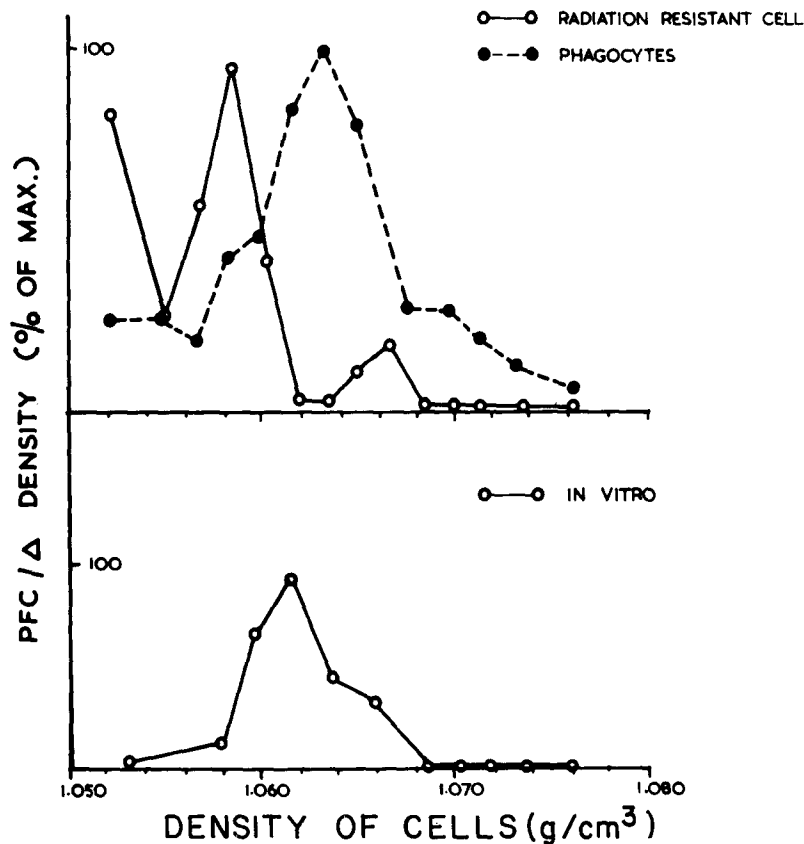


FIG. 4. Comparison of the radiation resistant stimulatory cell profile with phagocytic cell profile. Fraction 3 cells (8×10^6 /culture) were added to 3×10^6 cells of different densities (given 1000 rads) and cultured with SRBC. The irradiated cells gave no response alone. (CBA mice). The in vitro response is shown for comparison.

cell was in the density range less than 1.060 g/cm^3 , with a smaller peak of activity in the dense region of the gradient.

Attempts to obtain stimulation of fraction 3 by fraction 1 across a nucleopore membrane (pore size 0.8μ) were not successful, although other stimulatory effects described later in the paper were obtained across such a membrane. It was tentatively concluded, therefore, that cell-cell contact was necessary for the activity of this component in fraction 1.

It was also observed that addition of fraction 1 (1000 rad) caused aggregation of cells in populations which otherwise formed an even monolayer. This was readily observed in a microadaptation of the Marbrook tissue culture in which the cells were grown on Millipore filters. (Auerbach, 1968, private communication). Dr. Auerbach observed that fraction 3 cells alone did not form cell aggregates, while a mixture of fraction 3 and fraction 1 (1000 rad) formed many aggregates. Similarly, thymus cells formed no clusters unless fraction 1 (1000 rad) was added.² Further examination of the latter system showed the production of large pyroninophilic cells in such a culture, while none were

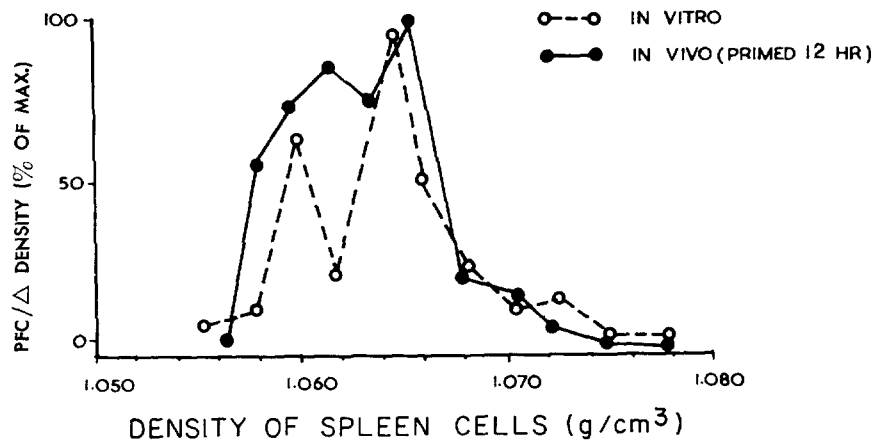


FIG. 5. Comparison of in vitro and primed in vivo profiles. Normal spleen cell fractions from F₁ hybrid mice were assayed in vitro. Spleen cells from F₁ mice which had been primed with SRBC 12 hr previously were fractionated and assayed in vivo.

formed by either component alone. The possible significance of these observations will be discussed.

Relationship between fraction 2 cells and primed spleen cells: Density gradient analysis has demonstrated that mouse spleen cells contained two major populations of plaque-forming cell (PFC) precursors. Fraction 3 (Fig. 1) was the peak of the in vivo response and depended on the presence of cells in fraction 1 for activity in vitro. Fraction 2, by contrast, was much less dependent on the addition of fraction 1 for activity, and although it contained only a small proportion of the radiation resistant cell, (Fig. 4) it was in fact the peak of the in vitro response.

Another difference is illustrated in Fig. 2. Fraction 2 showed a linear dose-response curve in vivo, while fraction 3 showed an apparent exponential dose response.

² Haskill, J. S., Pauline Byrt, and J. Marbrook. Unpublished observations.

It was observed that the in vitro profile of responsiveness of normal spleen cells to SRBC was very similar to the in vivo profile of spleen cells taken from an animal which had been exposed to antigen within the previous 12 hr (17,

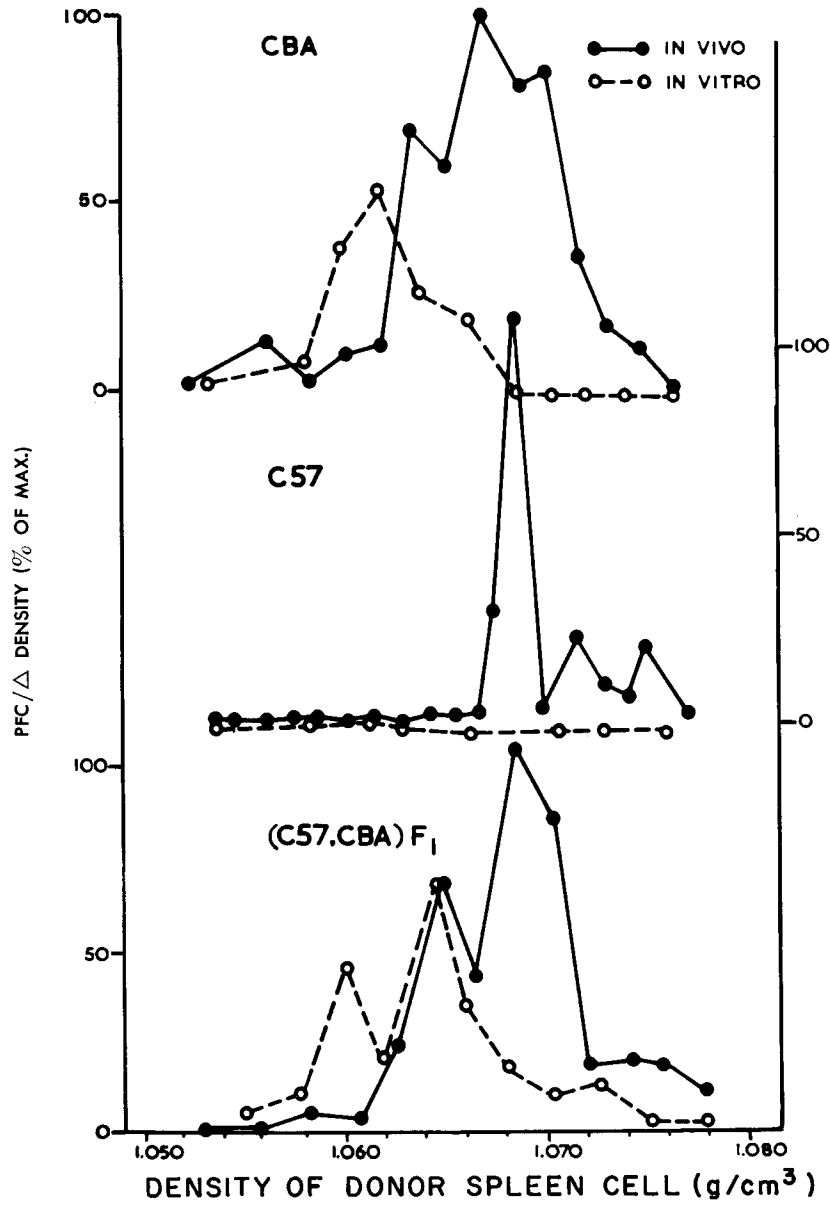


FIG. 6. Comparison of in vivo and in vitro profiles of CBA, C57BL, and F₁ hybrid mice.

18). The two profiles are shown in Fig. 5. This evidence suggested that the cells in fraction 2 may have been "primed" cells, and that the *in vitro* profile was the profile of that particular population of PFC precursors which had previously been exposed to antigen or otherwise stimulated into a state which more readily responded in culture.

TABLE II
Effect of In Vivo Priming on C57 and (CBA.C57) F₁ Spleen Activity In Vitro

Spleen cell source*		PFC/culture	Increase over primary response
(CBA.C57) F ₁	Untreated	670	
	12 hr after SRBC in vivo	1050	1.6
C57	Untreated	70	
	12 hr after SRBC in vivo	940	13.4

* Cells from untreated mice of the strain indicated, or from animals 12 hr after an injection of 10^9 sheep erythrocytes (i.v.) were prepared for tissue culture as usual, and cultures assayed for antibody-forming cells after 3 days *in vitro*. Standard deviation of the results was approximately 30%.

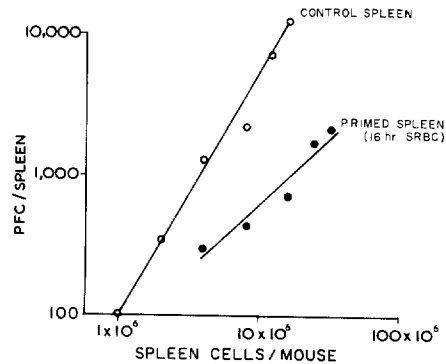


FIG. 7. Comparison of dose response of unfractionated normal spleen cells and unfractionated primed (16 hr after an injection of SRBC) spleen cells assayed *in vivo*. The slope of the control cells is 1.6, and of the primed cells 1.0 (log/log plot).

Absence of primed cells in normal C57BL mice: Attempts in this laboratory and others³ to obtain a primary immune response *in vitro* using C57BL spleen cells have not been successful, although an *in vivo* response was obtained. Density gradient analysis was carried out, therefore, on the *in vivo* profile of C57BL spleen cell responsiveness to SRBC to determine if there were any PFC

³ Diener, E. Private communication.

precursors in the fraction 2 region. Results are shown in Fig. 6, and for comparison, the profiles of CBA and (CBA.C57BL) F_1 spleen cells.

It may be seen that while the C57BL *in vivo* profile had peak activity in the

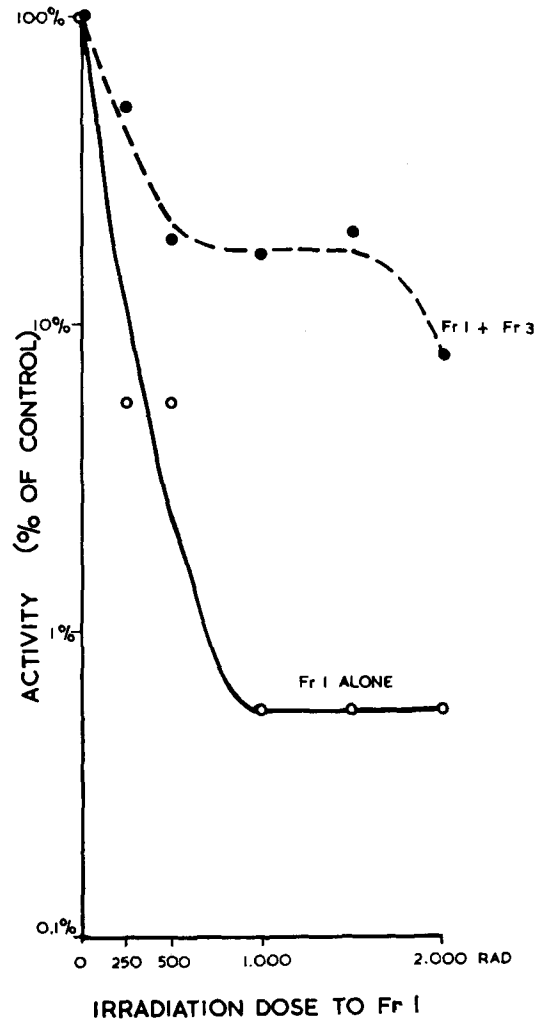


FIG. 8. Irradiation dose response of fraction 1, assayed alone and after mixing with fraction 3. Normal CBA spleen cells were fractionated, and fraction 1 irradiated with doses as shown. 5×10^6 fraction 1 cells were cultured alone or with 5×10^6 fraction 3 cells.

same density region as the CBA profile, there was virtually no activity in the lighter density regions. If the suggestion is correct that the *in vitro* response is largely due to primed cells, then priming of C57BL mice should have had a

much greater effect on the *in vitro* activity of the spleen cells than priming of either CBA or F₁ hybrid mice. The results of such a priming experiment are shown in Table II, and it is evident that 12 hr priming with SRBC *in vivo* had a profound effect on the *in vitro* responsiveness of C57BL spleen cells, while only marginally affecting the F₁ hybrid spleen responsiveness.

Dose response of primed and unprimed spleen cells in vivo: Fig. 2 illustrates that fraction 3, the region of major *in vivo* response, showed an exponential dose response curve *in vivo*, while fraction 2 showed a linear dose response. The dose response of normal spleen cells *in vivo* was also exponential (Fig. 7). If a cell in the donor inoculum or in the irradiated host was needed to initiate the immune response of PFC progenitors in fraction 3 or in the normal spleen cells, and if the linear dose-response of fraction 2 indicates that this initiatory cell is no longer required, then *in vivo* priming should also result in a decrease in cell interactions and a consequent decrease in the slope of the dose-response curve for unfractionated primed spleen cells. Fig. 7 illustrates that the *in vivo* dose response of unfractionated primed spleen cells is linear, and supports the suggestion that there exists in the spleens of CBA and C57-CBA mice a subpopulation of PFC progenitors which have already been exposed to antigenic stimulation.

Evidence for a Multi-Cell Phenomenon in the In Vitro Response of Spleen Cells to SRBC.—Spleen cells of density range 1.067–1.0695 g/cm³ (fraction 3) show an exponential dose-response curve when assayed *in vivo* (Fig. 2), suggesting that a multi-cell phenomenon is occurring in the response. There is also some evidence for a multi-cell phenomenon in the *in vitro* response of this fraction, in addition to the previously demonstrated enhancing effect of a radiation-resistant cell. The results in Table I show that X-irradiation of fraction 1 reduces, but does not eliminate, the interaction between fraction 1 and fraction 3, suggesting either that irradiation does have a partial effect on the radiation-resistant cell, or that fraction 1 consists of two components. In the latter case, one component, the radiation resistant cell, would stimulate fraction 3, while the second component might itself be stimulated by fraction 3 and in addition would be radiation-sensitive.

To examine this possibility, cells from fraction 1 were subjected to a range of X-ray doses between 250 and 2000 rad and then assayed *in vitro* either alone or after mixing with an equal number of fraction 3 cells (Fig. 8). The activity of fraction 1 alone showed a different radiation dose-response curve to that of the cell mixture. The activity of fraction 1 alone declines rapidly and is inhibited 95% by 500 rad and 99.5% by 1000 rad. The activity of fraction 1 (irradiated) plus fraction 3 shows an initial decline and then a plateau between 500 and 1500 rad. This curve was obtained by subtracting the number of plaques obtained when fraction 3 and fraction 1 (at the irradiation dose shown) were cultured separately from the response obtained when they were mixed.

It represents, therefore, the stimulation obtained on mixing the fractions, calculated as percentage of that obtained by mixing fraction 3 plus fraction 1 unirradiated. Table I (Experiment 2) shows the extent of stimulation obtained

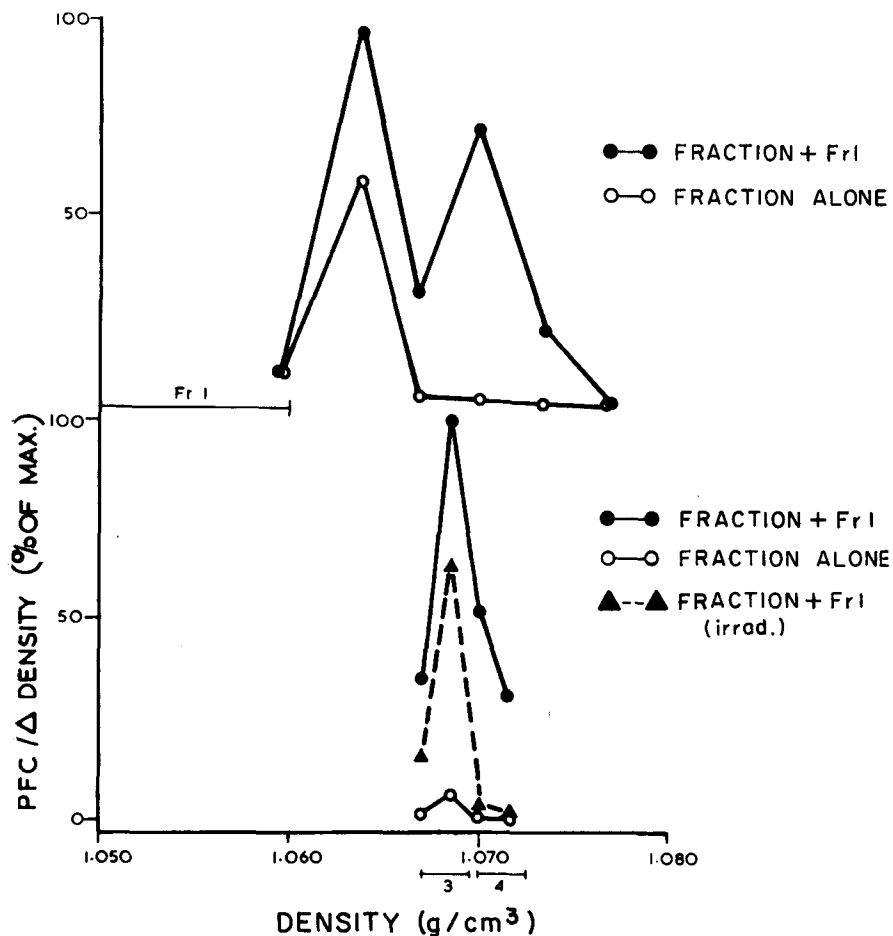


FIG. 9. Effect of fraction 1 and fraction 1 (1000 rad) on SRBC responsiveness of normal spleen cell fractions (CBA). In the experiment shown in the lower graph, 5×10^6 fraction 1 cells (irradiated and nonirradiated) were added to 8×10^6 cells of the other spleen cell fractions and assayed in vitro. Fig. 3 is shown again at the top for comparison.

by mixing fractions 1 and 3. This again suggests that there is not only a radiation-resistant component of fraction 1 which stimulates fraction 3, but also a radiation-sensitive component, itself stimulated by a component of fraction 3.

Evidence for a Second Stimulatory Cell Using Specific H2-Antisera.—Fig. 9

also illustrates the stimulation obtained when fraction 3 and fraction 1 cells, irradiated or nonirradiated were mixed, but special interest has been directed to fraction 4 which is not stimulated at all by irradiated fraction 1 cells, but apparently stimulates, or is stimulated by, unirradiated fraction 1 cells. Analy-

TABLE III
Identification of PFC Precursors

Spleen cell source	PFC/culture*		
	Assay in the presence of serum		
	Normal CBA	Anti CBA	Anti C57
(CBA.C57) F ₁ Fr 1 †	20	0	0
CBA Fr 4	14	0	20
F ₁ Fr 1 + CBA Fr 4	140	0	24

* Normal spleen cells from (CBA.C57) F₁ mice and CBA mice were fractionated on separate BSA density gradients. Fractions were prepared as indicated and cultured in vitro 3 days. Three assays were performed on each culture, in the presence of either normal serum or antiserum as indicated.

† Fr, fraction.

TABLE IV
Comparison of In Vitro Activity of Thymus and Bone Marrow Cells with Fraction 1 and Fraction 4 Spleen Cells

Cell type*	Number of cells/culture ($\times 10^{-6}$)	No. PFC/culture
Fr 1 †	5	63
Fr 4	10	6
Thymus	10	0
Bone marrow	5	0
Fr 1 + fr 4	5 + 10	492
Fr 1 + thymus	5 + 10	207
Fr 1 + bone marrow	5 + 5	54
Fr 4 + thymus	10 + 10	21
Fr 4 + bone marrow	10 + 5	3

* Fractions 1 and 4 were prepared after fractionation of normal CBA spleen cells on a BSA density gradient.

† Fr, fraction.

sis of the stimulation occurring when fraction 1 and fraction 4 cells are mixed, using anti-H2 sera showed that the PFC precursor cells were all derived from fraction 1 (Table III). In this experiment, fraction 1 cells were derived from F₁ hybrids, and fraction 4 cells from CBA donors. In this way any nonspecific stimulation due to a mixed lymphocyte reaction would be in the direction of stimulating only the fraction 4 cells (Dutton, 19). Anti-CBA serum inhibited the PFC of both CBA and F₁ hybrid type, while anti-C57BL serum inhibited

only PFC of F_1 hybrid type. It is concluded, therefore, that the PFC precursors in fraction 1 may be stimulated by a component of fraction 4 which is presumably also present in the neighboring fraction 3.

Activity of Thymus Cells to Stimulate Fraction 1.—The SRBC responsiveness of fraction 1 may be stimulated by fraction 4 and also by thymus cells, but not by bone marrow cells (Table IV). The thymus is not regarded as a source of PFC precursor cells (20). The stimulation of fraction 1 by both thymus and fraction 4 has been observed to occur when the two cell populations were separated by a membrane, either nucleopore (pore size 0.8μ) or dialysis. The effect, however, was variable, and usually about half of that obtained when the cells were in contact.

TABLE V
Effect of Irradiation on Fraction 4

	Spleen cell type	Number of Cells/culture ($\times 10^{-6}$)	PFC/ culture
Exp. 1	Fr 1*	0.5	<2
	Fr 4	4.0	20
	Fr 1 + Fr 4	0.5 + 4	112
	Fr 4 (1000 rad)	4.0	<2
	Fr 1 + Fr 4 (1000 rad)	0.5 + 4	2
Exp. 2	Fr 1	2.5	44
	Fr 4	8.5	17
	Fr 1 + Fr 4	2.5 + 8.5	276
	Fr 4 (1000 rad)	8.5	<2
	Fr 1 + Fr 4 (1000 rad)	2.5 + 8.5	64

* Fr, fraction.

As a further test of the action of these "thymus-like" cells, they were subjected to a dose of 1000 rad *in vitro* and tested for their ability to enhance fraction 1 cells. The ability of fraction 4 to stimulate fraction 1 was inhibited by this treatment, suggesting that either cellular proliferation or long-term maintenance of this cell was needed for enhancement (Table V).

It was also observed that fraction 4, obtained from $(C57BL \times CBA)F_1$ mice, had no stimulatory effect when added to fraction 1 from either CBA or F_1 , although fraction 1 from F_1 mice was stimulated by fraction 4 from CBA mice (Table III).

In Vitro Profile of PFC Precursor Cell.—After the identification of a radiation-sensitive cell in fraction 1 and a radiation-sensitive cell in fraction 4, both of which stimulate the responsiveness of spleen cell fractions to SRBC, an attempt was made to determine the profile of the *in vitro* PFC-precursor. A small number (1×10^6 cells) of cells from a range of density-gradient fractions was added to a larger number of fraction 1 (1000 rad) and fraction 4 cells ($5 \times 10^6 +$

5×10^6). It was thought that under these conditions the PFC precursors would be the limiting cells, and so the profile obtained would represent that of the precursor cells only. The mixture of fraction 1 (1000 rad) and fraction 4 cells alone gave a very low response, and this was subtracted from that of the response of the mixture plus the added cell fraction before the profile calculations were made. A typical profile obtained in this way is illustrated in Fig. 10.

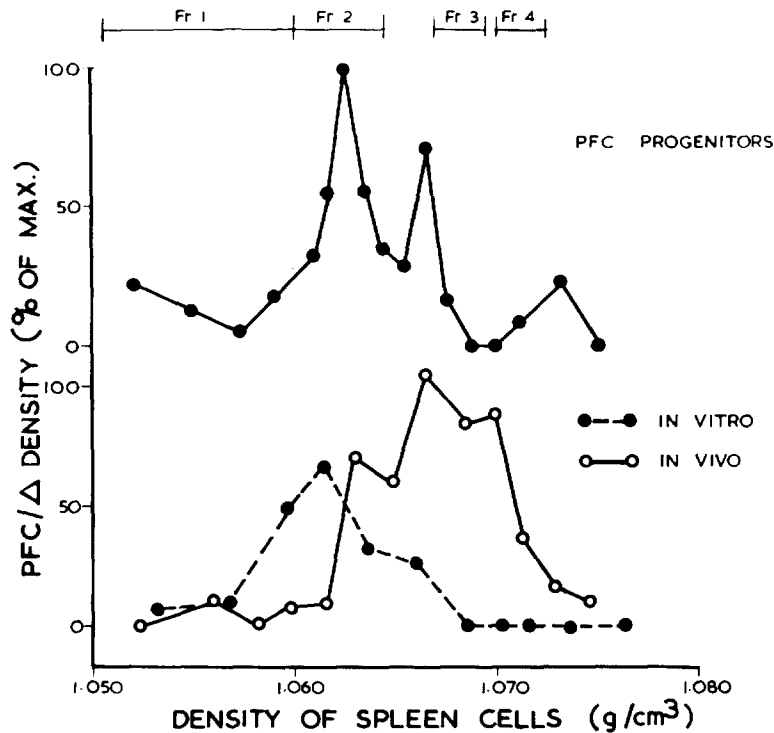


FIG. 10. PFC Progenitor Profile. Normal CBA spleen cells were fractionated. 5×10^6 fraction 1 (1000 rad) plus 5×10^6 fraction 4 cells were added to 1×10^6 cell fractions and assayed in vitro with SRBC. The CBA in vitro and in vivo profiles are shown for comparison.

There is activity in most density regions which corresponds with the in vivo response. The one notable exception is in the region of fraction 3. It should be noted also that this is the density region of maximal in vivo activity of the C57BL mice.

DISCUSSION

The demonstrations by Mishell and Dutton (5), and more recently by Marbrook (7), of the initiation of a "primary" response to erythrocyte antigens in suspension cultures of spleen cells, offer convenient methods for an analysis

of the cellular events involved in an immune response. A necessary prerequisite to furthering these studies is a demonstration that the immunological processes occurring *in vivo* can be satisfactorily reproduced *in vitro*. The primary aim of this report is to detail the results of a comparison of the adoptive transfer culture method (8-10) with that of the suspension culture method of Marbrook (7). The present study was also initiated with the hope of demonstrating immunologically significant interactions between distinct cell types as provided by separation of spleen cells by equilibrium density gradient centrifugation (6, 17, 18).

The data presented herein suggest that there are at least three distinct cell types involved in the *in vitro* primary immune response of CBA spleen cells. The possibility of such a complex situation has recently been suggested by Mosier and Copplestone (21). These three cell types have been classified as a result of direct comparisons between the *in vivo* and *in vitro* culture methods. They are (a) light density radiation-resistant cell, apparently not a phagocytic cell, (b) a radiation-sensitive cell, detected in the denser region of the gradient, and (c) the PFC precursor cell. It was not possible from the data obtained to conclude whether a phagocytic cell was involved in the *in vitro* response. They cannot be excluded since phagocytic cells are present in the highest numbers in the region of maximum *in vitro* response (Fig. 2). In addition, the lack of correlation between the profile of the radiation-resistant cell and that of the phagocytes does not necessarily indicate that the radiation-resistant cell is not a phagocytic cell. It may represent a small subpopulation of phagocytic cells with a specialized function.

Recent work by Mosier (22) has suggested another possible role for this cell and is related to the observations that fraction 1 (irradiated) causes aggregation of cell populations which otherwise show no tendency to aggregate. Mosier has shown that the association of mouse spleen cells in clusters was essential for the primary immune response *in vitro*. No response was obtained in the absence of clusters. It is suggested, therefore, that the irradiation-resistant cell may have an important role to play *in vitro* by effecting the aggregation and thus the close association of the cells required for the response. The significance of the interaction that occurs *in vitro* between the radiation-sensitive, thymus-like cell in fraction 4 and the antibody-forming cell precursor is difficult to assess. If this same interaction occurs *in vivo*, the *in vivo* profile could be a composite of the profiles of the thymus-like cell and the PFC progenitor, and this may explain why the *in vivo* profile does not correspond exactly with the profile for PFC precursors (Fig. 10), and may also explain the exponential dose-response curve of the *in vivo* response.

It is not possible from the present data to eliminate the possibility that either of the interactions described is a "feeder-layer" effect, and that one of the cells is simply supplying some nutrient lacking in the culture medium. The fact that the thymus-like effect can be mediated across a dialysis membrane suggests

that the *in vitro* action of thymus cells may be simply a humoral one and not the same as has been demonstrated *in vivo* (14, 23).

In addition, comparisons of *in vivo* and *in vitro* techniques are complicated by the fact that the *in vivo* environment is very complex, and such factors as recirculation of cells, recruitment of cells, the structural organization of the spleen, and the different homing efficiencies of cells of different size may all play additional roles. None of the present data show the induction of PFC's by the mixing of two populations which separately have zero activity, and so an absolute requirement of either of the auxiliary, cells has not been demonstrated.

It has been suggested that the *in vitro* profile provides evidence for the presence of primed PFC progenitor cells. That is, cells which have in some manner already been exposed to antigen. Another possible explanation is that fraction 2 works *in vitro* because there is an excess of the thymus-like cell in this region, and that this excess of one of the interacting cells explains the linear dose response. The priming effect (Fig. 4) may represent an altered density distribution of the thymus-like cell, i.e. a depletion in the dense regions and an increase in the lighter density regions. If this is true, then the effect of priming on the C57BL mouse *in vitro* activity could represent an influx of such cells into the spleen or the transformation of some precursor cell. Within 10 hr of antigen stimulation in the rat, resting immune-competent cells from regions three and four are stimulated into cell cycle which is reflected by a decrease in density to that of fraction 2. (17, 18). Globerson has reported that nonrespondent cells could be made to respond *in vitro*, provided that they were first stimulated with the mitogen PHA (24, 25). It may be that the process described here as priming is in reality not an immune phenomenon, but rather a tissue culture requirement for cycling cells.

An important question which remains unanswered after this investigation is whether or not the *in vitro* response is measuring the same response as is the adoptive transfer assay. Certainly, a significant portion of the response, especially at low cell numbers, comes from the primed cell populations. As the numbers of cells per culture are increased, so are the possibilities of cell interactions. One of these, fraction 4 + fractions 1 and 2 leads to an increase in the primed response. The other fraction 1 and fraction 3 leads to what appears to be a primary response. At present it is not possible to know which of these latter two interactions gives the greatest number of antibody-forming cells in a culture of unfractionated spleen cells. Until this is known, it may not be feasible to study such phenomena as antigenic competition in *in vitro* cultures.

SUMMARY

The BSA density-gradient technique for separating mouse spleen cells into partially purified populations has been used to compare the responsiveness of such populations to SRBC using *in vivo* and *in vitro* techniques. Two major

populations were distinguished, one of which responded very well in vivo with an exponential dose response and poorly in vitro (fraction 3), and another which responded in vivo and in vitro with a linear dose response (fraction 2). A light density, radiation-resistant component was identified which markedly stimulated the response of fraction 3 in vitro, and a density gradient profile was obtained for this cell which did not correspond with a macrophage profile. A high density, radiation-sensitive cell was identified which stimulated the response of PFC precursors in lighter regions of the gradient. The activity of this cell could be replaced using thymus cells. A density profile for the PFC precursor cell was obtained by assaying small numbers of spleen cell fractions in the presence of an excess of the two auxiliary cell types.

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