

THE ENHANCING EFFECT OF BONE MARROW CELLS ON THE  
PRIMARY IMMUNE RESPONSE OF THE ISOLATED  
PERFUSED SPLEEN\*

BY ROBERT C. ATKINS,† M.B., WILLIAM A. ROBINSON, M.D., AND BEN  
EISEMAN, M.D.

(From the Departments of Surgery and Medicine, Colorado University, Denver,  
Colorado 80220)

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Recent studies on cellular interaction in the immune response have postulated that two or perhaps three cell types are needed for antibody synthesis. Globerson and Auerbach (1) reported that both thymus and bone marrow cells were necessary to restore the ability of spleen fragments from lethally irradiated mice to initiate a graft-vs.-host reaction in vitro. Claman et al. (2) presented evidence that a mixture of thymus and bone marrow cells produced far more hemolytic antibody-forming cells in irradiated recipients than either cell type alone. Mitchell and Miller (3) concluded that the immediate precursor of the 19S hemolysin-forming cells was bone-marrow derived, and that thymus or thoracic duct lymphocytes recognize antigen and interact with it in some way that triggers off differentiation of the essentially passive bone marrow-derived precursor cell to a specific antibody-forming cell. Mosier and Coppleson (4) suggested that the response to a single antigen may involve at least two and possibly three antigen-specific cells. Radovich, Hemingsten, and Talmage (5) have demonstrated synergism between cells of the bone marrow or spleen and antibody-forming cells.

We have recently shown that the isolated spleen, perfused and oxygenated, at normal temperature can be primarily stimulated in vitro to form antibody-producing cells. This preparation provides an excellent model for study of immune mechanisms and cellular interaction. Unlike previous in vitro studies (6-8), the structural integrity of the entire spleen is maintained intact. Recirculation of cells occur as in vivo and may allow for necessary cellular interaction within the proper architectural framework.

The aim of our experiments has been to produce large numbers of antibody-producing cells in a system that perhaps could be adapted to human organs for use in clinical immunotherapy. In initial experiments, however, the yield of antibody-forming cells from the isolated perfused spleen alone has been low.<sup>1</sup>

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† Present address: University of Washington School of Medicine, Department of Medicine, Division of Nephrology, Seattle, Washington 98105.

Address for reprints: Dr. B. Eiseman, Department of Surgery, Denver General Hospital, West Sixth Ave. and Cherokee St., Denver, Colorado 80200.

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The present studies were done to determine whether this response could be enhanced by addition of bone marrow or peripheral blood cells to this system and to assess the role of various cellular components in the immune response. It has been shown that addition of bone marrow or peripheral blood lymphocytes to the medium perfusing the isolated spleen increases the number of antibody-forming cells produced after primary antigenic stimulation *in vitro*.

#### Materials and Methods

Splenectomy was performed in twenty-five 12-15 kilogram anesthetized Hampshire pigs under aseptic conditions. After cannulation of the splenic artery, the spleen was washed free

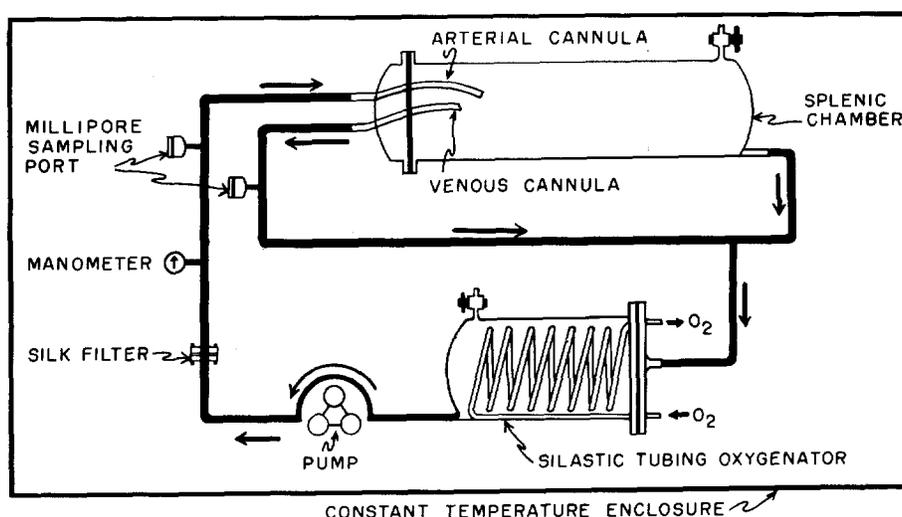


FIG. 1. Diagram of splenic perfusion circuitry.

of blood with 1.5 liters of 0.9% sodium chloride containing 5000 units heparin, 60 mg procaine hydrochloride, and 7 mEq sodium bicarbonate per liter. The washout procedure required 5-10 min, after which the spleen was placed in a sterile glass perfusion apparatus of original design (9).

*Perfusion Circuitry (Figs. 1 and 2).*—The main elements of the perfusion system were a perfusate filled perfusion chamber containing the cannulated spleen, a membrane oxygenator, and a constant flow pump. The complete apparatus was contained in a constant temperature enclosure. All components were of siliconized (Siliclad, Clay-Adams, Inc., New York) pyrex glass and the tubing was medical grade silastic, thus avoiding the cytotoxic properties of some synthetic materials (10).

The *perfusion chamber* was a 12 inch  $\times$  2½ inch glass cylinder. The chamber top incorporated arterial and venous connections which were splinted to the requisite cannula by a cuff of silastic tubing. The chamber had two outlet ports at the distal end, one for air release, the other for drainage.

The *membrane oxygenator* is photographed in Fig. 3. 50 ft of 0.125 inch (outer diameter  $\times$  0.078 inch (inner diameter) Silastic tubing is wrapped in two layers around concentric glass

baskets. This unit fits over a central hollow glass core which is part of the outer casing. Connections for oxygen inflow and outflow are incorporated in the oxygenator top. Perfusate enters through the center of the top and leaves the chamber at its distal lower end. A stopcock for air release is situated at the distal end of the chamber. The priming volume of the oxygenator is 250 ml and it has the potential to deliver 6.5 ml of oxygen per minute. It is easily cleaned, reassembled, and is steam autoclavable.

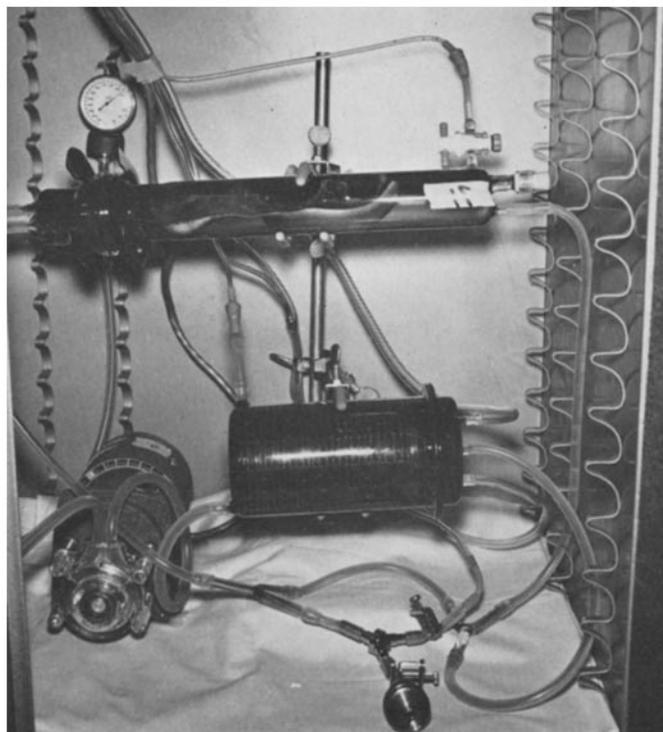


FIG. 2. Photograph of splenic perfusion apparatus, within its constant temperature enclosure.

A *filter* of nylon within a glass casing was imposed between the oxygenator and the arterial cannula. All tubing was  $\frac{5}{16}$  inch (outer diameter)  $\times$   $\frac{3}{16}$  inch (inner diameter) Silastic tubing (Dow Corning, Corp., Englewood Cliffs, N.J.) and the "Y" connections were of glass.

*Sampling ports* were protected from bacterial contamination by Millipore filters (0.45  $\mu$ ) (Millipore Corp., Bedford, Mass.).

*Perfusion* of the oxygenated tissue culture medium perfusate was by Model 7015 Cole-Palmer constant flow pump (Cole-Palmer Instrument Co., Chicago, Ill.).

*Perfusate* was McCoy's 5A medium, to which was added 10% decomplexed (56°C for 30 min) pooled pig serum (Colorado Serum Co., Denver, Colo.). Antibiotics (penicillin 100 units/ml and neomycin 40  $\mu$ g/ml) were added to ensure sterility. The perfusate volume was 1200 ml, and final concentrations were: Na<sup>+</sup>, 154 mEq/liter; K<sup>+</sup>, 4.8 mEq/liter; Ca<sup>++</sup>, 2.1 mEq/liter; glucose, 300 mg/100 ml, pH 7.1; osmolality, 302 milliosmoles/kg.

*Bone marrow addition:* After splenectomy and the establishment of splenic perfusion, both femur and humeri were aseptically excised from the same donor pig. The lower epiphysis of each bone was sawn off. The marrow was flushed from the bones using heparinized (10,000 units/liter), buffered (7 mEq sodium bicarbonate/liter), 0.9% sodium chloride. The bone marrow cell suspension was allowed to stand for 30 min, fat rising to the surface, bone and debris settling to the bottom. The intermediate cell suspension was drawn off and washed three times in McCoy's 5A medium by centrifugation at 1200 revolutions per minute for 10 min. Nucleated white cells were counted in a hemocytometer and added to the perfusing medium 3-4 hr after commencement of perfusion.

*Collection of peripheral white cells:* Nucleated white cells were separated by a modification of the method of Coulson (11).

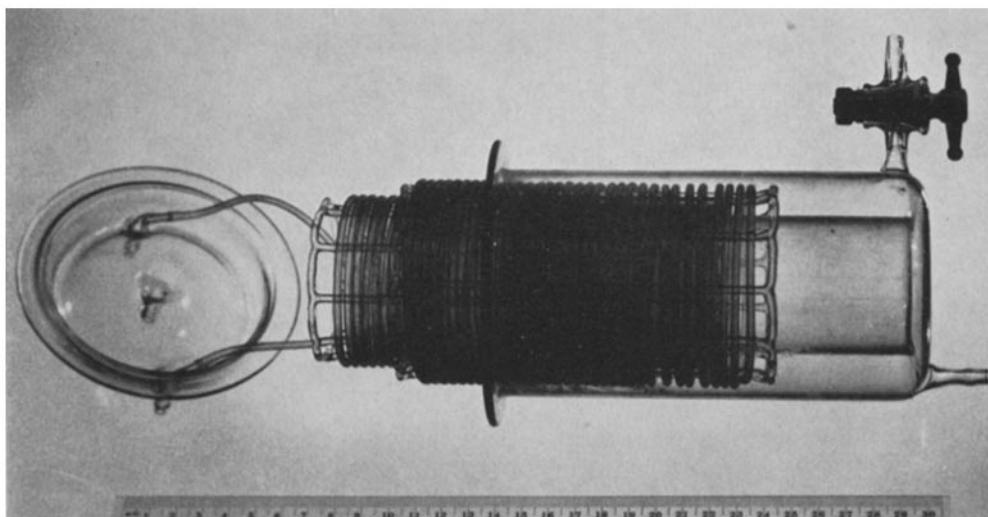


FIG. 3. Photograph of partially assembled membrane oxygenator.

After splenectomy, 500 ml of blood was collected from the donor pig into a heparinized vacuum flask (Travenol Laboratories, Inc., Morton Grove, Ill.) by intracardiac puncture. The blood was transferred to measuring cylinders, 3% gelatin added, and the red cells allowed to settle at 37°C. The supernatant was withdrawn, passed through a column of packed 40-denier nylon, and produced a cellular suspension in which approximately 90% of the nucleated white cells were lymphocytes. The cells were washed three times in McCoy's medium and added to the splenic perfusing medium 4 hr after commencement of perfusion. The procedure was performed aseptically.

*Antigen:* 15 min after perfusion began, 0.05 ml of washed 50% sheep red blood cells (Colorado Serum Co.) per gram splenic weight was added to the perfusate.

*Perfusion technique:* 19 spleens were perfused for 3 days. An additional six were unperfused controls.

The perfusate flow was 2 ml/gram splenic weight per minute. The arterial oxygen partial pressure (APO<sub>2</sub>) was maintained between 200 and 250-mm Hg. The pH was maintained

between 7.1 and 7.2 by the intermittent addition of sodium bicarbonate and alteration of the CO<sub>2</sub> gas concentration flowing through the membrane oxygenator. Glucose was added as required to maintain a level of 300 mg/100 ml. The original perfusion medium was not exchanged.

*Splenic irradiation:* After washout, the spleen was wrapped in a sterile towel and irradiated for 6.2 min, 6 inches from a <sup>60</sup>Co-source to give a total dose of 2000 R. This is sufficient to inhibit the capacity of immunocompetent progenitor cells to proliferate and differentiate into antibody-synthesizing cells (12). This dosage is lethal in mice.

*Antibody determinations:* The number of antibody-forming cells produced was estimated by the Jerne technique (13). At the end of a 3-day perfusion, cells were collected from the perfusing medium by centrifugation (1200 revolutions/min for 10 min) and washed three times in McCoy's medium. Splenic samples from various areas were minced to give a single cell suspension and washed three times in McCoy's medium. Cell viability was determined by trypan

TABLE I  
*Number of Hemolytic Plaques Produced by Cells from Unimmunized Pig Spleens and Following Unimmunized Normothermic Perfusion for 3 Days*

	Experiment	Plaques/10 <sup>7</sup> cells*	
		spleen	perfusate
Not Perfused	12	0 ± 0	—
	34	4.8 ± 1.6	—
	52	0.8 ± 0.4	—
	53	0.4 ± 0.5	—
Perfused	92	0.8 ± 0.4	1.6 ± 0.4
	126	0.5 ± 0.5	0.3 ± 0.5

Background level of hemolytic foci from unimmunized nonperfused and perfused pig spleens.

\* Each figure represents mean of five plates ± SD.

blue exclusion (14). Both viable and nonviable cells were included in the cells plated. A total of 10<sup>7</sup> cells were used on each plate. Five plates were averaged for each determination.

## RESULTS

Normal unimmunized spleens from most animal species contain small numbers of cells which give rise to hemolytic foci when tested by the Jerne plaque technique (background level). The number of such cells in spleens from unimmunized pigs is shown in Table I. The mean background level of 1.5 plaques/10<sup>7</sup> cells for four unimmunized pigs compares with previous studies.<sup>1</sup> Also shown in Table I is the number of plaques produced by unimmunized spleens perfused for 3 days. The background levels did not change.

Two pigs were primarily immunized in vivo with 5 ml 50% washed sheep red blood cells intravenously, the spleens removed after 3 days and tested for the number of hemolytic foci. The numbers were 1066 ± 184 and 2960 ± 160 per 10<sup>7</sup> cells.

At the completion of 3 days of isolated normothermic perfusion, the per-

centage of viable cells averaged  $30\% \pm 18.4$  within the spleen and  $57\% \pm 19.5$  of the circulating perfusate cells. In the irradiated preparations with added viable bone marrow cells the percentage splenic cell viability was re-

TABLE II  
*Effect of Isologous Bone Marrow and Peripheral Lymphocytes on the Antibody Response to Sheep Red Blood Cells of an Isolated Pig Spleen Perfused for 3 Days*

Experiment	Cells added per gram splenic weight $\times 10^{-6}$	Plaques/ $10^7$ cells	
		Spleen	Perfusate
A. Sheep RBC but no added cells:			
75	0	$29 \pm 7.8^*$	$7 \pm 3.6^*$
80	0	$13 \pm 4.5$	$4 \pm 0.5$
71	0	—	$78 \pm 27.6$
125	0	$16 \pm 7.1$	$29 \pm 9.0$
B. Sheep RBC + blood nucleated white cells:			
84	1	$13 \pm 1.3$	$8 \pm 1.4$
114	29.5	$292 \pm 74.0$	$575 \pm 121$
118	43.0	$56 \pm 1.0$	$206 \pm 39.3$
C. Sheep RBC + bone marrow nucleated white cells:			
85	7.9	$25 \pm 12.8$	$14 \pm 3.6$
89	11.4	$73.5 \pm 12.6$	$74 \pm 16.1$
95	5.9	$29 \pm 4.3$	—
96	32.0	$214 \pm 11.8$	$175 \pm 32.1$
112	16.8	$640 \pm 141$	$546 \pm 146$
115	31.5	—	$600 \pm 189$
116	50.5	$592 \pm 138$	$2962 \pm 631$
D. Sheep RBC + bone marrow cells + blood white cells:			
113	66.0†	$248 \pm 8.0$	$474 \pm 88.9$
E. Irradiated spleen + sheep RBC + bone marrow cells:			
120	29.6	$14 \pm 4.7$	$10 \pm 0.8$
124	25.7	$4 \pm 2.5$	$22 \pm 8.5$

Effect of isologous bone marrow and peripheral lymphocytes on the antibody response to sheep red blood cells of an isolated pig spleen perfused for 3 days.

\* Each figure represents mean of five plates  $\pm$  SD.

† Bone marrow cells, 24.0; blood lymphocytes, 42.0.

duced to  $17\% \pm 6.5$  and the perfusate cells to  $49\% \pm 1.0$  after 3 days of perfusion.

Table II shows the number of hemolytic plaques produced by splenic and perfusate cells from isolated spleens immunized in vitro with sheep erythrocytes and perfused for 3 days. When immunization was done without addition of peripheral blood or bone marrow cells, a mean of 19.3 antibody-forming

cells per  $10^7$  splenic cells, and 29.5 antibody-forming cells per  $10^7$  perfusate cells was obtained (Table II, section A). This approximates the number obtained after 3 days' perfusion in a previous study in which the response to duration of perfusion was quantitated.<sup>1</sup>

These numbers were not increased by the addition of small numbers ( $10^6$  gram splenic weight) of nucleated peripheral white blood cells (Table II, section B). When greater numbers of nucleated peripheral white blood cells were added, the number of antibody-producing cells in both the spleen and perfusing medium rose dramatically.

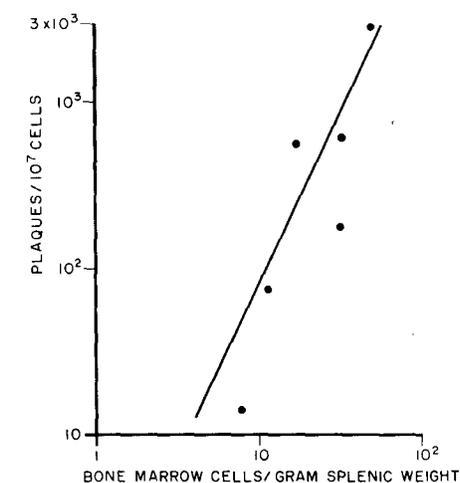


FIG. 4. Number of added bone marrow cells is plotted against the number of plaques/ $10^7$  perfusate cells on a log:log scale. A regression line with a slope of two is indicated. The plotted points approximate the line. This would suggest that two cell-types are involved in antibody formation.

Similar results were obtained when bone marrow cells were added to the medium (Table II, section C). Small numbers failed to enhance the response, but when larger numbers were added, the number of antibody-forming cells in both the spleen and the perfusing medium rose sharply.

When small numbers of bone marrow cells were added (experiments 85 and 95), there was no appreciable increase in the number of antibody-forming cells produced. Approximately twice this number of marrow cells (experiment 89) increased the number of antibody-forming cells obtained by a factor of two. With further increase in the number of bone marrow cells (experiments 96, 112, 115, 116), the number of antibody-forming cells produced was increased 20-fold as compared to antibody production when no marrow cells were added. With a 3-8-fold increase in marrow cells, the antibody response was increased by a factor of 10-20. This increase occurred both in the cells of the perfusate and within the spleen.

When the number of added bone marrow cells per gram splenic weight is plotted against the number of antibody-producing cells formed per  $10^7$  cells on a log:log scale, a regression line with a slope of two approximates the plotted points both for the perfusate (Fig. 4) and the splenic cells (Fig. 5). This suggests that at least two cell types are required for antibody formation.

Section D of Table II shows the effect of a combination of bone marrow and peripheral lymphocytes added to the system. A total of  $66.0 \times 10^6$  nucleated white cells per gram splenic weight were added (bone marrow  $24.0 \times 10^6$ ; peripheral blood lymphocytes  $42.0 \times 10^6$ ). The response was the same as when either cell type was administered alone.

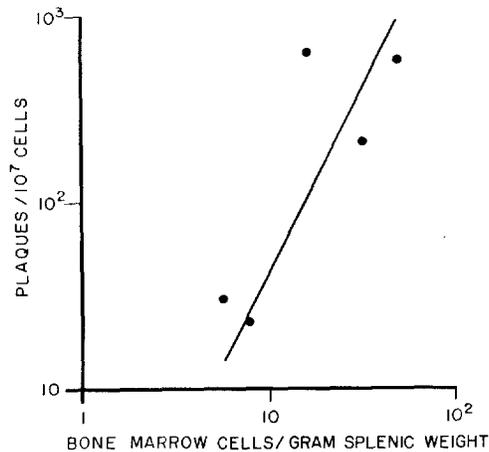


FIG. 5. Number of added bone marrow cells is plotted against the number of plaques/ $10^7$  splenic cells on a log:log scale. A regression line with a slope of two is indicated. The plotted points approximate the line. This would suggest that two cell types are involved in antibody formation.

Section E of Table II demonstrates the effect of prior irradiation of the isolated spleen on antibody production. Nonirradiated isologous bone marrow cells were added to the medium perfusing the irradiated spleen. The subsequent antibody response was minimal. The number of antibody-forming cells was only 50% of that obtained by the splenic cells alone (section A, Table II). It appears that both splenic cells and marrow cells are needed for an enhanced response.

#### DISCUSSION

The use of an ex-vivo isolated perfused spleen has provided an environment which can be easily controlled and yet closely simulates the in vivo situation. Here cells are recirculated through the spleen as in vivo, perhaps providing a structural frame facilitating cellular interaction necessary for the immune response.

The present studies have accomplished two objectives: (a) enhancement of

the primary immune response in the isolated perfused spleen with resultant facility to produce and collect larger numbers of antibody-producing cells, and (b) further demonstration of the interaction of various cell types in the immune response.

The results confirm synergism between bone marrow and splenic cells (2, 5). The nature of the enhancement phenomenon noted here is not entirely clear. We have not demonstrated conclusively that this is due to stimulation of uncommitted bone marrow or peripheral white blood cells rather than an increase in antibody-producing cells from the spleen itself, but this is most likely. This is particularly suggested by the poor antibody response when bone marrow or peripheral blood cells were added to irradiated spleen and by the rise in antibody-forming cell numbers as increasing numbers of bone marrow and blood cells are added to the system.

It is of interest that in the present studies addition of either bone marrow or peripheral lymphocytes resulted in enhancement of a similar magnitude. The enhancement accomplished by addition of bone marrow may be explained by the findings of Mitchell and Miller (3) who have suggested that the bone marrow cell is the actual effector cell and that thymic or thoracic duct lymphocytes recognize the antigen and are the memory cells. Presumably the spleen contains both cell types and the addition of bone marrow adds unprimed cells capable of forming antibody once the necessary cell has interacted with the antigen. Perhaps the small but definite bone marrow cell response was permitted by the survival after irradiation of some splenic memory cells.

The application of a log:log regression line to establish the proportion of interacting cell types has been used previously (4, 16). The regression line for both circulating and splenic cells conform to a slope of two which agrees with the concept that at least two cell-types are needed for antibody production.

It is more difficult to correlate the effect of the peripheral lymphocytes. It may be a function of the increase in number of recirculating cells within the perfusate. Ford and Gowans have shown that the number of lymphocytes entering the spleen influences the magnitude of an immune response initiated in the spleen (15). In our experiments peripheral lymphocytes could probably be regarded as recirculating thoracic duct cells. The pig has a unique lymph drainage system in that there is a great paucity of lymphocytes in the thoracic duct. It was suggested by Binns that lymph recirculation occurs at a local level and that efferent lymph channels enter the blood stream close to their origin (17).

Of more practical importance is that if a splenic preparation is to be used as an *in vitro* source of antibody, then marrow cells or peripheral lymphocytes should be added to the perfusing medium to gain optimal response.

#### SUMMARY

The addition of bone marrow cells or peripheral lymphocytes to the isolated pig spleen markedly enhanced the primary antibody response after 3-day perfusion and antigenic challenge *in vitro*. The splenic preparation without added cells or with the addition of marrow cells to an irradiated spleen gave a

limited response. Contributory evidence is provided that at least two distinct cell types are needed for antibody production. For optimal antibody response by an isolated perfused spleen, marrow cells or peripheral lymphocytes should be added to the system.

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