

IMMUNIZATION OF DISSOCIATED SPLEEN
CELL CULTURES FROM NORMAL MICE*,†

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This paper describes an in vitro system for the study of the factors that control and regulate the cellular events in the immune response. It was reported earlier (1) that dissociated cell suspensions obtained from the spleens of normal mice could be immunized in vitro to heterologous erythrocytes. The response is primary in the sense that it follows the first experimental exposure to the erythrocyte antigen. The response was measured by the increase in the number of hemolytic plaque-forming cells and by assay of antibody in the culture supernatants. The critical conditions for culture and immunization of the spleen cells included low oxygen tension, gentle agitation of the cultures, the inclusion of fetal bovine serum in the medium, adequate spleen cell density, and daily feeding of the cultures with a nutritional mixture.

This paper presents a more detailed account of the experimental system and some comparisons between in vitro and in vivo responses under a variety of experimental conditions. The in vitro response closely parallels that seen in vivo with respect to size, early kinetics, effect of antigen dose, and the inhibitory effect of passive antibody. These findings encourage the belief that observations made with the in vitro system are relevant to our understanding of the in vivo response. The in vitro differs from the in vivo response in that it appears to show a greater capacity to discriminate between different homologous erythrocyte antigens and that it shows no limitation or termination of the increase in 19S antibody-forming cells 4 or 5 days after the initiation of the response. These differences have been characterized in the hope that their further study may uncover underlying regulatory mechanisms involved in the response.

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Materials and Methods

Mice.—Hybrid mice from C57BL/6 female \times DBA/2 male (B6D2F₁) were used in all experiments, except where otherwise stated. Most were obtained from Simonsen Laboratories, Gilroy, Calif. Others were from our own colony, bred from C57BL/6 females and DBA/2 males obtained from Jackson Laboratories, Bar Harbor, Maine. Mice were 2–6 months old at the time of use. Mice of the same age and sex were used in individual experiments.

Biological Reagents.—Fetal bovine serum was obtained from several suppliers (Microbiological Associates, Bethesda, Md.; Grand Island Biological Co., Grand Island, N. Y.; and Reheis Chemical Co., Division of Armour Pharmaceutical Co., New York, N. Y.), but serum from the Colorado Serum Co., Denver, Colo., was used in most of the studies. Sheep and goat red cells, suspended in Alsever's solution, were obtained from the Colorado Serum Co. Burro red cells were obtained from the Davis Laboratories, Davis, Calif. Mouse serum was prepared from C57BL/6J and CAF₁ mice. Warmed mice were bled from the tail, the blood was allowed to clot and was incubated at 37°C for 1 hr, after which the serum and clot were separated by centrifugation. The serum was sterilized by passage through prewashed Millipore filters (2) and stored at 4°C. Fresh guinea pig serum was used as a complement source and was absorbed with sheep erythrocytes by the method of Kabat and Mayer (3). The absorbed guinea pig serum was frozen in 2 ml aliquots and thawed just prior to use. "Early" mouse antisera to sheep erythrocytes were obtained from B6D2F₁ mice immunized with a single injection of 0.2 ml of 10% sheep erythrocytes 5 days before bleeding; "late" mouse antisera to sheep erythrocytes were obtained 10–14 days after the last of three or more intravenous injections given at weekly intervals.

Chemical Reagents.—Balanced salt solution (BSS) was prepared as follows: dextrose, 1000 mg; KH₂PO₄, 60 mg; Na₂HPO₄·7H₂O, 358 mg; phenol red, 10 mg; CaCl₂·2H₂O, 186 mg; KCl, 400 mg; NaCl, 800 mg; MgCl₂·6H₂O, 200 mg; MgSO₄·7H₂O, 200 mg to 1000 ml in distilled water. Eagle's minimal essential medium, suspension salts, was obtained from Microbiological Associates (No. 12-126) and 100 ml volumes were supplemented with 200 mM glutamine (Microbiological Associates, No. 17-605F), 1 ml; 100 \times minimal essential medium nonessential amino acids (Microbiological Associates, No. 13-114), 1 ml; and 100 mM sodium pyruvate (Microbiological Associates, No. 13-115), 1 ml. In addition, medium was supplemented with serum according to the particular experimental design. Medium with 5% fetal bovine serum was routinely employed.¹ Eagle's minimal essential medium, suspension type, modified by excluding sodium bicarbonate and adjusting the pH to 7.2 with 1 N NaOH, was obtained from Grand Island Biological Co. for use as a cell suspension preparative solution and for inclusion in a nutritional mixture (Gibco No. 109S).

The nutritional mixture for daily feeding of the cultures was made as follows:

Essential amino acids (50 \times concentrated, Eagle, Microbiological Associates, No. 13-606), 5 ml. Nonessential amino acids (100 \times concentrated, Eagle, Microbiological Associates, No. 13-114), 2.5 ml. Glutamine, 200 mM (Microbiological Associates, No. 17-605F), 2.5 ml. Dextrose, 500 mg. Minimal essential medium, suspension, Eagle, modified without NaHCO₃ (Grand Island Biological Co., No. 109S), 35 ml. The pH was adjusted with 1 N NaOH to 7.2, and 7.5 ml of 7.5% NaHCO₃ were added. The mixture was sterilized by passage through washed cellulose filters (Millipore, GS type).

Agarose was obtained from L'Industrie Biologique Francaise S.A., Gennevilliers, France.

Glassware.—It was found advisable to prerinse all glassware in 0.85% saline for 30 min at 37°C prior to use. The glassware was then rinsed in glass-distilled water and autoclaved.

Preparation of Cell Suspensions.—Mice were killed by cervical dislocation. The spleens were removed using sterile techniques and placed in a tissue culture grade, sterile disposable

¹ Fetal bovine serum contains antigens which cross-react with sheep erythrocyte antigens (see below).

60 mm Petri dish (Falcon Plastics, Los Angeles, Calif., No. 3002) containing 5–10 ml of either sterile BSS or modified Eagle's minimal essential medium without bicarbonate. The spleens were teased apart with mouse tooth forceps by gently tearing the capsule and releasing the cells. The suspended cells were transferred to a sterile 12 ml tube. It was found advisable to suspend the spleens of no more than six mice in a single 12 ml tube to avoid the formation of gelatinous clots which occurred if the cell suspension was too dense. The tubes were placed in an ice bath for 3–5 min to allow particles to settle, and the suspension of dissociated cells was transferred to a graduated conical tube, centrifuged for 10 min at 4°C and 1000 rpm. After centrifugation, the supernatant fluid was removed and the pellet resuspended in culture medium at the desired concentration. If the packed cell volume was diluted 1:80, the final cell concentration was approximately 2×10^7 per milliliter. An aliquot of the suspended cells was taken for assay of the cell number, and the cell suspension adjusted to a final cell density of $1.5\text{--}2.0 \times 10^7$ per milliliter.

Standard Cell Culture Conditions.—1 ml volumes of spleen cell suspension (1.5×10^7 per milliliter) were incubated in 35 mm Petri dishes (Falcon Plastics, No. 3001). The immunizing erythrocytes were washed three times in 15 volumes of sterile BSS and 30 μ l of a 1% suspension (approximately 3×10^6 cells) added to the appropriate dishes. The dishes were placed in boxes and were incubated at 37°C at an atmosphere of 7% O₂, 10% CO₂ and 83% N₂. The boxes were placed on a rocker platform (Bellco Glass, Inc., Vineland, N. J., No. 6700) which rocked back and forth at 7–10 complete cycles per minute. The cultures were fed each day with 75–100 μ l of the nutritional mixture and 30 μ l of fetal bovine serum. The culture conditions were modified as described below in the experiments where particular culture conditions were investigated.

Cell Harvest and Hemolytic Plaque Assay.—At the time of harvest, the cells were resuspended by gentle agitation and by scraping the dish with a plastic policeman. The cell suspensions from two or three dishes were pooled and the cells centrifuged at 1000 rpm for 10 min at 4°C. The number of plaque-forming cells was assayed by a modification of the technique described by Jerne et al. (4) which measures 19S antibody-forming cells. The cells were resuspended in an appropriate volume and several dilutions were made to ensure that some contain 20–80 plaque-forming cells per 25 or 100 μ l for the hemolytic plaque assay. 25 or 100 μ l of cell suspensions were added to tubes containing 0.5 ml of a 0.5% agarose solution in BSS and 50 μ l of a 7% suspension of sheep erythrocytes maintained at 42°–44°C. The tubes were quickly mixed and poured on the surface of a microscope slide previously coated with 0.1% agarose. The agar set in 5–10 min and the slides were transferred to racks, complement (1/10 guinea pig serum) added and then incubated for 3 hr at 37°C. The plaques were counted using a hand magnifying glass and a strong indirect light source. Slides were not stained.

Duplicate assays were made at each of several dilutions to ensure that slides were obtained with 20–80 plaques per slide for accurate plaque counts.

Periodically during the course of this work, plaques were examined microscopically and a single cell was always found in the center of each.

The variation between replicate cultures was examined in these experiments and the results presented in Table I. The data presented represents the mean of duplicate assays done on several single cultures. The variance between replicate cultures is less than two-fold; a difference of greater than two-fold is considered significant.

RESULTS

In Vitro Response to Sheep Erythrocytes.—

Spleen cell suspensions obtained from a pool of normal isologous mice were incubated under the standard culture conditions described in Materials and Methods and 3×10^6 sheep erythrocytes were added at time 0. The number

of hemolytic plaque-forming cells rose more or less exponentially from 1 per 10^6 at time 0 to around 1000 per 10^6 recovered cells at 4 days. A typical experiment is illustrated in Fig. 1 and the results of 20 consecutive experiments

TABLE I
Variation between Replicate Cultures Obtained in Three Separate Experiments

Experiment No.	PFC/ 10^6 recovered cells assayed on day 4		
381	725	1103	775
386	1175	1094	1138
388	584	527	480

The figures represent the means of duplicate assays on cell suspensions obtained from single culture dishes. The numbers indicate the number of PFCs per 10^6 recovered cells. Comparable cell recoveries were obtained from replicate cultures within an experiment.

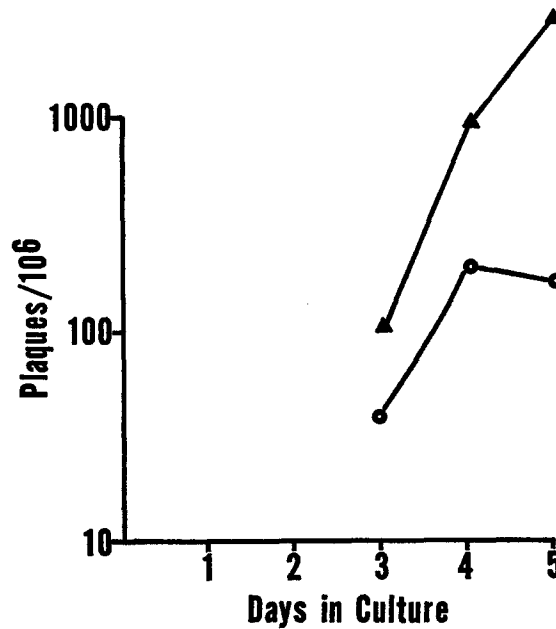


FIG. 1. Response of normal mouse spleen cell suspensions to sheep erythrocytes. Spleen cell suspensions were cultured with (▲—▲) and without (○—○) 3×10^6 sheep erythrocytes. The number of PFCs per 10^6 recovered cells was assayed on days 3, 4, and 5. Comparable cell recoveries were obtained in stimulated and control cultures at each day.

are listed in Table II. The figure and the table also record the number of plaque-forming cells seen in the absence of added sheep erythrocytes and the number of cultured cells recovered at harvest.

The following points can be noted:

1. Successful immunization occurred in all 20 experiments.

2. Significant rises (up to 100-fold) in the number of plaque-forming cells (PFCs) occurred in the absence of added sheep erythrocytes.
3. In most instances (10 out of 12), the number of PFCs in antigen-stimu-

TABLE II
In Vitro Immunization of Mouse Spleen Cells with Sheep Erythrocytes

Experiment No.	Day 4				Day 5			
	PFC per 10 ⁶		Cell recovery		PFC per 10 ⁶		Cell recovery	
	Control	Stim.	Control	Stim.	Control	Stim.	Control	Stim.
293	99	967	48	48	38	1200	42	36
294	200	900	—	—	175	2960	42	40
296	46	1314	41	41	29	3514	36	34
297	81	2317	47	38	124	3885	33	32
299	373	9140	46	40	120	7636	32	36
303	110	1035	38	42	—	—	—	—
307	52	280	25	40	—	—	—	—
308	249	2000	—	—	—	—	—	—
309	16	1050	29	31	—	—	—	—
310	165	1885	25	38	301	2960	28	28
312	83	359	30	30	40	900	—	—
314	31	1107	38	38	—	—	—	—
315	36	503	36	38	—	—	—	—
317	52	1090	37	44	24	870	27	27
320	14	245	42	40	—	—	—	—
321	54	675	50	56	6	870	48	44
324	105	750	44	44	48	1050	44	44
328	94	2500	29	23	260	6210	24	27
329	73	1054	38	50	86	1550	33	32
330	130	925	38	33	—	—	—	—
Average...	103	1505	38	40	104	2800	35	35
Range...	14-373	280-9140	25-50	23-56	6-301	870-7636	24-48	27-44

The data recorded represents the results obtained from 20 consecutive experiments. The numbers missing from the series are of experiments with different protocols. Four experiments in which contamination occurred are also omitted.

The number of PFCs per 10⁶ recovered spleen cells and the per cent cell recovery in cultures with (stimulated) and without (control) sheep erythrocytes were determined on day 4 and day 5.

0.3-3 PFCs per 10⁶ were present at time 0.

lated cultures was higher on day 5 than on day 4, while in unstimulated cultures the reverse was true.

4. The cell recoveries were quite constant, generally falling between 35 to 45% of the planted cells. No differences were seen in cell recovery between stimulated and unstimulated cultures.

5. While there was considerable variation in the number of PFCs observed from experiment to experiment, 80% of the sheep erythrocyte-stimulated cultures assayed at day 4 and 100% of those assayed at day 5 fell within a fourfold range of the respective means, or 8–12 doublings of the initial value for the 4 day cultures and 9–13 doublings for the 5 day cultures.

6. The culture fluids were examined for hemolytic antibody for several of the sheep erythrocyte-stimulated 5 day cultures, and titers of 1:16 to 1:64 were found. No antibody was found in cultures grown without sheep erythrocytes.

Variation of Culture Conditions.—

Gas phase: Comparisons were made of sheep red cell-stimulated cultures made from single pools of spleen cells in 7, 21, and 40% oxygen. The highest responses were found with 7% O₂, but the differences between 7 and 21% were not significant. The data in Table III show the results of three experi-

TABLE III
Comparison of Response to Sheep Erythrocytes at Two Oxygen Tensions

Experiment No.	7% O ₂	40% O ₂
386	1135	15
391	575	55
1290	334	15

The data represents the number of PFCs per 10⁶ recovered cells assayed at day 4. Comparable cell recoveries were obtained from different cultures within each experiment.

ments comparing 7 and 40% oxygen. In each case, the 40% oxygen was strongly inhibitory.

Motion: Comparisons were made between cultures rocked at approximately 7 cycles per minute and others which remained stationary. The comparisons were made in both 7 and 21% oxygen atmospheres. In most cases, the rocking cultures were significantly better, developing two to four times as many plaque-forming cells, but significant immunization did occur in stationary cultures.

Spleen cell concentration: The effect of spleen cell concentration was studied, making 1 ml cultures with different numbers of spleen cells from single pools of suspended mouse spleen cells. All cultures contained approximately 3 × 10⁶ sheep red cells added at time 0. The number of PFCs was assayed on day 4. The results of four experiments are shown in Table IV. The optimal response measured as PFCs per 10⁶ cells was found to be given by 1–2 × 10⁷ spleen cells per milliliter. 5 × 10⁶ cells per milliliter gave a suboptimal result in every case and 3 × 10⁷ per milliliter frequently developed proportionately fewer plaque-forming cells.

Fetal bovine serum: One source of the variation observed in Table I was

found to be differences in the fetal bovine serum employed. Several lots were obtained from four suppliers; some of these gave very poor results. The data presented in Table V show the results of comparisons between three lots of fetal calf serum in two experiments. Cultures were made from a single pool of suspended cells, and all were stimulated with sheep red cells at time 0. The

TABLE IV
Effect of Spleen Cell Concentration on the Response to Sheep Erythrocytes

Initial cell concentration per ml	Experiment No.			
	337	349	355	361
2.5×10^6	—	—	—	1100
5×10^6	1220	200	500	2125
1×10^7	6400	600	1300	4500
2×10^7	7300	1000	1100	1020
3×10^7	5900	1000	700	530

The data represents the number of PFCs per 10^6 recovered cells assayed at day 4.

TABLE V
Comparison of the Ability of Various Lots of Fetal Bovine Serum to Support the Response of Mouse Spleen Cultures to Sheep Erythrocytes

Fetal bovine serum*	Experiment No.		
	328	329	336
R129	2500	1054	—
M62025	650	300	—
M62026	535	460	—
CSC162	—	1730	4300
CSC170	—	—	4300

The results represent the number of PFCs per 10^6 recovered cells at day 4.

* R129: Rehatuin, Reheis Chemical Co. M62025, M62026: Fetal bovine serum, Microbiological Associates. CSC162, CSC170: Fetal bovine serum, Colorado Serum Company.

individual cultures were fed with the appropriate serum in the nutritional mixture. Two of the sera gave low results compared to the third. It was observed that some lots of sera contained factors, presumably antibody, which caused complement-dependent lysis of mouse red cells. Some of these antibody-containing sera gave "normal" in vitro responses, but they have not been routinely employed. The stability of the calf serum has not been investigated. Sera have been stored frozen, but have been maintained at 4°C after thawing.

Antigens in the fetal bovine serum which cross-react with sheep erythrocytes:

As noted above, there is a significant rise in the number of plaque-forming cells which occurs in the absence of added sheep erythrocytes. It is believed that this rise is the consequence of substances present in the fetal bovine serum which cross-react with sheep erythrocyte antigens, as discussed below.

It was found that some mouse anti-sheep erythrocyte sera show a line of precipitation with a protein of β mobility present in the fetal bovine serum on immunoelectrophoresis.

Antigen.—

Red cell dose: The effect of antigen dose was assessed by making several cultures from a single pool of cells and adding various amounts of sheep red cells at time 0 as shown in Table VI. In all experiments, a marginal increase in plaque-forming cells developed with 1×10^4 sheep red cells; significant but partial responses were observed with 1×10^5 cells, and optimal results were

TABLE VI
Effect of Sheep Erythrocyte Dose on the Response of Mouse Spleen Cell Cultures

Sheep erythrocytes added	Experiment No.			
	299	308	350	353
None	380	250	36	45
10^3	450	—	—	130
10^4	800	325	150	220
10^5	3200	1120	1225	825
10^6	9150	2000	2900	1060
10^7	8550	2300	2200	—

The data represents the number of PFCs per 10^6 recovered cells assayed at day 4.

observed with 1×10^6 erythrocytes. Antigen dose experiments with in vivo primed spleen cells (see below) gave qualitatively similar results; optimal responses were observed with 10^6 or more sheep cells, but significant responses were seen with as few as 10^3 erythrocytes in this more sensitive system.

Immunization with red cells from other species: In addition to sheep red cells, successful in vitro immunization has been obtained with goat, burro, cattle, horse, pig, and rabbit red cells. Goat and cattle red cells showed significant cross-reactivity with sheep red cells and had significant numbers of plaque-forming cells when cultures were stimulated only by antigens in the fetal bovine serum. Horse red cells also showed the rise with fetal bovine serum. Burro red cells show very little cross-reactivity with sheep red cells and appeared to have no cross-reactivity with antigens in the fetal calf serum, since no rise in the background to burro cells was observed. Pig erythrocytes were found to be unstable and prone to nonspecific lysis, while rabbit erythrocytes were unsatisfactory as indicators in the hemolytic plaque assay. Dog, cat, and

ferret red cells were also tried, but proved to be unsuitable for the plaque assay in our hands. Since our main interest was in having suitable antigens for studies involving cross-reactions or lack of cross-reactions, goat and burro cells were selected for more extensive study.

The data in Table VII show the results of experiments in which cultures from single pools of mouse spleen cells were made and were cultured alone or immunized with sheep red cells, goat red cells, and both sheep and goat red cells. The greatest number of plaque-forming cells developed when homologous cells were used for immunizing and testing. There was significant cross-reactivity between the goat and sheep red cells, as shown by the fact that the heterologous red cells always stimulated more plaque-forming cells than fetal

TABLE VII
Comparison of Cultures Immunized with Sheep and Goat Erythrocytes

Immunizing red cells	Experiment No.			
	362		397	
	Red cells used in assay			
	SRC	GRC	SRC	GRC
Control	30	37	138	30
Sheep erythrocytes (SRC)	1720	152	1740	162
Goat erythrocytes (GRC)	116	350	770	1078
Sheep and goat erythrocytes	1430	615	2380	575

Results in PFCs per 10^6 recovered cells assayed at day 4. Singly immunized cultures received 3×10^6 erythrocytes; doubly immunized cultures received 3×10^6 of each kind of erythrocyte.

calf serum alone. Fetal calf serum stimulated significant numbers of plaque-forming cells against both antigens. Finally, when cultures were immunized with both antigens, an optimal response (within the limits of the assay) was observed when tested against each red cell.

Similar experiments comparing sheep and burro red cells are shown in Table VIII. Significant immunization only occurred in cultures stimulated with the antigen being tested. There was no evidence of cross-reactivity between the red cells, since the number of plaque-forming cells in heterologous stimulated cultures did not differ from cultures stimulated with fetal calf serum alone. Control cultures stimulated only with fetal bovine serum did not develop significant numbers of plaques when tested against burro cells. Cultures immunized with both antigens responded optimally to each; the slight differences observed are not significant.

Sheep red cell variation: It became evident that certain lots of sheep red

cells resulted in relatively low *in vitro* stimulation compared to most others. A large number of blood samples from single sheep was obtained to investigate this phenomenon. Blood samples were screened and classified as H for high stimulation and L for low stimulation. Cultures from a single pool of mouse spleen cells were then immunized *in vitro* with either H or L type erythrocytes. The results are shown in Table IX. Cultures stimulated with H cells and tested against H cells developed approximately four times as many PFCs as cultures stimulated with L cells and tested against L cells. When the cultured cells were assayed against the opposite type of blood, the results were uniformly low. When cultures were immunized against both types of cells simultaneously (Experiment 357), the response was that of an H type immuni-

TABLE VIII
Comparison of Cultures Immunized with Sheep and Burro Erythrocytes

Immunizing red cells	Experiment No.					
	369		370		386	
	Red cells used in assay					
	Sheep	Burro	Sheep	Burro	Sheep	Burro
Control	87	<1	13	<1	41	4
Sheep	1130	<1	590	<1	1135	3
Burro	12	160	22	232	53	282
Sheep and burro	900	185	575	215	905	220

Results in PFCs per 10^6 recovered cells. Singly immunized cultures received 3×10^6 erythrocytes; doubly immunized cultures received 3×10^6 of each kind of erythrocyte.

zation when tested with H cells, showing that L cells were not toxic. If anti-H cells were assayed against a mixture of H and L red cells, the plaque count approached the H level, but approximately three-fourths of the plaques were hazy, containing residual unlysed cells, as was seen when cultures immunized with sheep cells were tested against a mixture of sheep and goat red cells. When H-stimulated cultures were tested against several lots of H type cells, the plaque counts were indistinguishable and, conversely, when either type of culture was tested against several lots of L type cells the results were indistinguishably low.

The phenomenon of H and L type sheep cells had not been observed in routine testing of *in vivo* immunity. In order to investigate the capacity of *in vivo* immunity to distinguish between the two types of cells, groups of mice were immunized intravenously with 0.2 ml of a 10% suspension of either H or L red cells which had previously been classified by *in vitro* immunization.

Dissociated cells from individual mice were obtained 4 days after immunization and assayed against H and L type red cells separately. The results of these experiments are shown in Table X. The mice appeared to react equally against H and L cells and the immune cells gave equivalent numbers of PFCs when tested against either the immunizing or opposite red cell.

Both L and H type sheep red cells have been found among four breeds of sheep. We have been unable to associate the H-L difference with the R-O blood type system.

Effect of Passive Antibody.—

In these experiments, either the sheep erythrocytes used as antigen or the mouse spleen cells were preincubated in various dilutions of mouse anti-sheep

TABLE IX
Detection of Variation in the in Vitro Response of Mouse Spleen Cell Cultures to Sheep Erythrocyte Suspensions Obtained from Different Sheep

Immunizing red cells	Experiment No.							
	350		353		355		357	
	Red cells used in assay*							
	H	L	H	L	H	L	H	L
H	2900	395	1060	240	1100	400	2200	400
L	495	350	195	225	250	350	500	500
H and L	—	—	—	—	—	—	2250	550

The data represents the number of PFCs per 10^6 recovered cells assayed on day 4.

* "H" represents a batch of sheep erythrocytes that elicited a high response and "L" a batch that elicited a low response.

erythrocyte serum for 1 hr at room temperature. In either case, the treated cells were then washed three times before placing in culture. Two types of mouse anti-erythrocyte sera were used: (a) "early," obtained 5 days after a single injection of 0.2 ml of a 10% erythrocyte suspension; and (b) "late," obtained 10–14 days after the last of three or more injections. The antisera were not further characterized in the present study. Further experimental details are indicated in the footnotes to Fig. 2 and Table XI.

It can be seen (Fig. 2) that treatment of the red cell antigen with late antiserum completely abolished its ability to elicit a response in all but the highest dilutions of antiserum. The early antiserum was only effective at the highest concentration. The suppressive effect of the late antiserum was somewhat dependent on erythrocyte concentration in that some stimulation was obtained when the red cell dose was increased 10- or 100-fold over that normally found to give a maximal stimulation.

In contrast, little or no suppression of the response was observed when the mouse spleen cell suspension was pretreated with the antiserum (Table XI). The same late antiserum was used in both experiments and early antisera were not tested by incubation with spleen cells.

Continuation of the Response Beyond Day 4.—

In vivo, the number of plaque-forming cells declines after day 4 (4). We have observed increases in vitro on day 5 and occasionally on day 6, suggesting that the mechanism which acts in vivo to terminate the response may not operate in vitro. However, since the culture conditions deteriorate during the 4th to 6th day, it was necessary to initiate the response in vivo in order to study it at later times.

Experiments with in vivo primed cells: When cells were taken from mice at

TABLE X
Lack of Variation in the In Vivo Response to Sheep Erythrocyte Suspensions Obtained From Different Sheep

Immunizing red cells	Red cells used in assay							
	H	L	H	L	H	L	H	L
H	1100	1500	780	1250	1820	2300	1280	1250
L	1000	1150	1300	1550	1730	1460	1660	1620

The data represents the number of PFCs per 10^6 spleen cells obtained from mice 4 days after an in vivo injection of 0.2 ml of 10% packed sheep erythrocytes. H and L erythrocytes refer to the same erythrocyte suspensions indicated in Table IX.

the height of the response, i.e. 4 days after immunization, and were cultured with added antigen, a very marked increase in plaque-forming cells was observed. Such cells will be referred to as in vivo-primed cells. Comparisons were therefore made between cultures of in vivo-primed cells and in vivo-boosted animals, as shown in Table XII. In these experiments, several animals were immunized with 0.2 ml of 10% sheep red cells intravenously. 4 days later, some of the animals were given a second intravenous injection of the same dose of red cells (boosted animals). Some of the animals were saved for later testing (nonboosted animals) and the remainder were sacrificed and the spleens used for making a single pool of suspended in vivo primed cells. The suspended cells were cultured, one group with 3×10^6 sheep red cells and the other in the absence of added red cells. The latter cultures cannot be considered to be unstimulated, however, since the fetal bovine serum contained in the medium contains cross-reacting red cell antigens, as will be discussed below. After 3 and 4 days, both types of culture and the spleens of boosted and control mice were assayed. Control, unboosted mice, which

TABLE XI
Effect of Coating Mouse Spleen Cells with Anti-SRC Serum

Spleen cells	Antigen	Experiment 296	Experiment 315
Normal	FBS only*	46	36
"	Normal SRC	1314	503
"	Coated SRC	40	51
Coated	Normal SRC	824	775

The data represent the number of PFCs per 10^6 recovered cells assayed on day 4. Comparable cell recoveries were obtained in all cultures. 2% sheep erythrocytes were coated with 1/50 late antiserum and 10% spleen cell suspensions were coated with 1/20 late antiserum as described in the text.

* Control cultures containing only fetal bovine serum antigens.

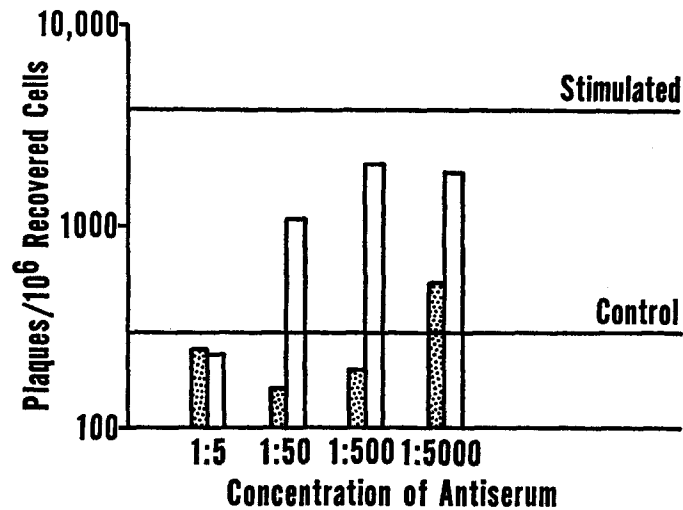


FIG. 2. Effect of concentration of antiserum used for treating sheep erythrocytes. The heights of the bars represent the number of PFCs per 10^6 recovered cells assayed on day 4. Comparable spleen cell recoveries were obtained in all cultures. The closed bars represent the response to erythrocytes treated with late antisera and the open bars the response to erythrocytes treated with early antisera. The upper line represents the response to untreated erythrocytes and the lower line the control in the absence of erythrocytes.

had received only a single injection 7 or 8 days before, showed a rapid fall in plaque-forming cells, as has been reported by many investigators. Spleens from mice which had been boosted with a second intravenous injection of sheep red cells showed either a slight rise or maintenance of the level of plaque-forming cells seen at the time of the second antigen injection for 3 to 4 days. In vivo-primed cells cultured without added erythrocytes, but in the presence

of fetal bovine serum, showed a slight increase in plaque-forming cells. Cells cultured with sheep erythrocytes showed a strikingly large increase in plaque formers, up to 40 times as many plaque-forming cells as we have ever observed in *in vivo* immunized mice.

The results of three experiments in which the 4-day-primed cells were grown in medium containing either fetal bovine serum or normal mouse serum are shown in Table XIII. These results show that the response of *in vivo* primed cells to further antigen occurs nearly as well in normal mouse serum as in fetal bovine serum, though the cultures grown without red cells show a marked decline in normal mouse serum, closely resembling the results obtained with

TABLE XII
Responses of 4 Day Primed Cells

Group	Experiment No.				
	286	292	295	368	390
1. <i>In vivo</i>	130	370	—	—	120
2. <i>In vivo</i> boosted	540	1,125	—	—	2,120
3. <i>In vitro</i> control	3,200	2,400	1,600	1,630	2,706
4. <i>In vitro</i> + sheep erythrocytes	24,000	16,000	45,000	127,000	71,925

In each experiment a group of mice were immunized with a single intravenous injection of 0.2 ml of 10% sheep erythrocytes on day -4. At day 0 mice were treated in one of four ways: group 1 left untreated; group 2 reinjected with the same dose of antigen; the mice in groups 3 and 4 were killed and spleen cell suspensions were incubated with (group 4) or without (group 3) 3×10^6 sheep erythrocytes.

The figures represent the number of PFCs per 10^6 , spleen cells (groups 1 and 2) or recovered cells (groups 3 and 4) assayed on day 4. Comparable cell recoveries were obtained in groups 3 and 4 in individual experiments.

the nonboosted animals. This is in contrast to experiments with cells obtained from unimmunized mice and grown with sheep erythrocytes in normal mouse serum. Such cultures have never shown a response. The data in Table XIV, showing the responses of cells from animals immunized 4 days before culturing and grown in normal mouse serum, indicate that maximum responses occur to 1×10^6 sheep erythrocytes. The response of primed cells is more sensitive than that of normal cells, in that significant responses could be detected to as few as 1×10^3 sheep cells.

DISCUSSION

The data presented clearly show that dissociated cells obtained from the spleens of normal, unimmunized mice can be successfully immunized *in vitro*. The events represent a primary response in the sense that they follow the

first experimental exposure to the specific antigens, although presumably the mice have encountered environmental cross-reacting antigens (5). The present system differs from those described by most other workers in that it utilizes dissociated cells and the response is of comparable magnitude to that which occurs during primary *in vivo* immunization.

Fishman and his colleagues have described the response of rat lymph node cells and lymph node fragments to extracts of peritoneal cells previously incubated with antigen (6-8). It is not clear whether the activity of such extracts depends on their content of highly active antigenic derivatives (9, 10) or on RNA carrying specific biosynthetic information derived from the peritoneal cell donor (11). In either case, the amount of antibody synthesized is small, relative to that obtained on *in vivo* immunization. Globerson and Auerbach

TABLE XIII
Responses of 4 Day Primed Cells Cultured in Normal Mouse Serum

Culture conditions	Experiment No.		
	368	374	390
FBS	1,630	324	2,706
FBS + sheep erythrocytes	127,000	28,750	71,925
NMS	37	18	70
NMS + sheep erythrocytes	37,500	32,600	24,800

Mice were injected with 0.2 ml of 10% sheep erythrocytes on day -4. Spleen cell suspensions were prepared on day 0 and incubated in medium containing either fetal bovine serum (FBS) or normal mouse serum (NMS) in the presence or absence of 3×10^6 sheep erythrocytes. The figures represent the number of PFCs per 10^6 recovered cells assayed on day 4. Comparable cell recoveries were obtained from different cultures within each experiment.

(12) have successfully immunized mouse spleen cell fragments to sheep erythrocytes. While the organ culture system that these authors employed makes cellular quantitation difficult, the system is advantageous for studying topographical distribution of precursor cells in the spleen and for culture studies of long duration since their fragments continued to synthesize antibody for more than 30 days. It is of some interest that Globerson and Auerbach found a requirement for oxygen far in excess of that which we found to be inhibitory. Presumably a steep oxygen gradient develops between the surface and interior of such fragments and the functional cells are thus protected from the inhibitory effects of high oxygen tension.

Saunders and King (13) have reported synthesis of antibody to coliphage in fragments, using a system similar to that of Globerson and Auerbach. They found, however, that it was necessary to culture spleen and thymus fragments together, which has not been the experience of other workers. Tao and Uhr

(14) have also described primary immunization to lymph node fragments using rabbit lymph node and ϕ X174 bacteriophage antigen. Bussard (15) has recently reported successful immunization of peritoneal exudate cells localized in gel during the period of cultivation. Such a system would be of major usefulness in studying clonal development of immune cells. Unfortunately, he was unable to immunize spleen cells or lymph node cells under similar conditions and the work is difficult to evaluate since it was not possible to perform the adequate negative controls.

Workers utilizing tissue fragments have stressed the desirability and perhaps obligatory requirement for preservation of the original tissue architecture. While such preservation may be useful in promoting cell interactions (if in-

TABLE XIV

The Effect of Erythrocyte Dose on the Response of Cultures of 4 Day In Vivo-Primed Cells

Sheep erythrocyte dose	Day of assay	
	3	4
0	23	18
10^3	190	610
10^4	1,330	4,186
10^5	4,925	10,761
10^6	13,066	32,600

Mice were injected with 0.2 ml of 10% sheep erythrocytes at day -4. Spleen cell suspensions were prepared on day 0 and cultured in medium containing normal mouse serum and varying numbers of sheep erythrocytes.

The figures represent the number of PFCs per 10^6 recovered cells assayed on days 3 and 4. Comparable cell recoveries were obtained from each culture on a given day.

deed they are necessary), the data we have presented shows that either such interactions are not always necessary or they can be achieved with the use of dissociated cells cultured in motion and with a high cell density.

The fact that the total response we observed is comparable in magnitude to that which occurs during primary in vivo immunization suggests that the same biological pathways may operate in each case. The data presented on the response to different sheep red cells, however, does raise the possibility that there may be important qualitative differences between the two reactions (see discussion below).

Much of the data presented refers to the conditions for culturing and immunizing spleen cells. The present conditions are arbitrary, both in the sense that they were developed empirically and in that other conditions may be found which will be more satisfactory. Nonetheless, it has been found so far that deviations from the conditions described most frequently result in a

gross reduction or even ablation of the response. While the critical conditions of the culture system, such as high cell concentration, low oxygen atmosphere, gentle agitation, daily feeding, and the need for fetal calf serum reflect biological requirements, we have no data to distinguish between the various hypotheses which could be generated concerning each condition. Thus, 40% oxygen is inhibitory to the development of plaque-forming cells, but whether this is due to inhibition of cell proliferation, as has been shown with some cells (16), or whether inhibition of differentiation or some other mechanism is involved, has not been determined.

A marked rise in the number of plaque-forming cells to sheep erythrocytes occurs in the absence of added erythrocytes. We have concluded that this probably represents a response to antigens present in the fetal bovine serum component of the medium and which cross-react with antigens present on the sheep erythrocyte. The relevant observations in support of this conclusion may be listed as follows: (a) Fetal bovine serum has been shown to contain high concentrations of the bovine erythrocyte antigen, J substance, which cross-reacts with R blood group antigens of sheep erythrocytes (17, 18). (b) Some (but not all) mouse anti-sheep erythrocyte sera precipitate with a protein of β mobility present in fetal bovine serum. (c) There is no comparable rise in plaque-forming cells against burro erythrocytes when normal spleen cells are cultured in fetal bovine serum (Table VIII). (d) Small amounts of fetal bovine serum (1%) stimulate large numbers of plaque-forming cells against sheep but not against burro erythrocytes when cells from in vivo-primed mice are cultured in fetal bovine serum.² Although the precise nature of the cross-reactivity has not yet been established, this explanation for the rise of plaque-forming cells in cultures without sheep cells appears very plausible.

Although successful responses of primed cells to further stimulation with sheep erythrocytes can be obtained with adult mouse serum instead of fetal bovine serum (Table XIII), it has not, as yet, been possible to demonstrate any "primary" response to sheep erythrocytes in mouse serum. It is possible that the presence of a soluble cross-reacting antigen in fetal bovine serum provides a necessary factor for some step only required in the primary response. The fact that responses to non-cross-reacting erythrocytes such as burros can be initiated makes this possibility unlikely, however, and this intriguing difference in the requirements for the primary and in vivo-primed response remains unexplained.

The data presented on antigen dose, immunologic specificity, and blocking of immunization by antisera show similarities between in vitro and in vivo immunization. With respect to antiserum inhibition of immunization, our

² Mishell, R. I. Unpublished observation.

findings that serum from hyperimmunized mice was more effective than early antiserum and that the inhibition depends on the combination of antibody with immunizing antigen is consistent with the data of most investigators (19–24). Rowley and Fitch have suggested another kind of antiserum-mediated inhibition, dependent on antibody reacting with immunocompetent cells (20). While we failed to find such inhibition (Table XI), our data is slight and differs from the experiments of Rowley and Fitch in that a different species was used and their observation was made with antiserum formed early in the immune response. More data will have to be obtained to explore further the possibility that various immunoglobulins may inhibit by different mechanisms.

We observed two striking discrepancies between *in vivo* and *in vitro* immunization. The *in vitro* experiments, showing an unexpected antigenic difference between two types of sheep erythrocytes which was not observed *in vivo*, suggest that the immunogenicity of particular antigens may be different in the two situations. It is possible that the excess number of plaque-forming cells that appear *in vitro* in response to some erythrocytes (H cells) compared to others (L cells) represent antibody-forming cells directed against one or more antigens not present or available on L cells. However, it is more likely that it is immunogenic *in vivo* but accounts for a relatively small part of the total response. If the latter interpretation proves to be correct, it may well be true that *in vitro* immunization develops to relatively few antigens, each of which causes a relatively larger response. While the explanation of this finding may well turn out to be trivial, it would seem wise to examine this difference further.

The second major difference between *in vitro* and *in vivo* immunization was observed with the responses of primed cells. In these experiments, the subsequent *in vitro* response far exceeded any response that has been observed *in vivo*. The work of Makinodan et al. (25) suggests that the response normally observed after secondary immunization can be accounted for by only 3% of the potentially competent cells and that regulatory mechanisms operate to limit the proliferation and/or differentiation of most of the cells. One interpretation of our data is that such control mechanisms are inoperative *in vitro* and that the potential of virtually all of the competent cells is expressed. The experiments presented provide no information on the nature of such regulatory mechanisms.

The system described exhibits a vigorous response to the first experimental exposure to antigen and utilized dissociated spleen cell suspensions. These two factors allow accurate quantitation of the cellular response and flexible experimental design which makes it useful for the study of the mechanisms controlling the immune response. The accompanying paper (26) describes an application of this system to the study of the role of proliferation in the response.

SUMMARY

A culture system for cell suspensions from mouse spleens has been described. The system provides adequate conditions for *in vitro* immunization on initial exposure to heterologous erythrocytes. The *in vitro* response closely parallels that observed *in vivo* with respect to size, early kinetics, antigen dose, and the inhibitory effect of passive antibody.

The response of cultured cells differs in two respects from that seen *in vivo*. There is an increase in the ability to discriminate between different varieties of homologous erythrocytes and the *in vitro* response does not appear to be limited by whatever mechanisms regulate the *in vivo* response.

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