A Requirement for Two Cell Types for Antibody Formation in vitro

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Table 3. Effect of 2-DOG administration on amounts of glycogen in liver and muscle. Two animals were used for each treatment, which was the same as that described in Table 2.

Compound administered	Glycogen	
	Liver (%)	Muscle (%)
None	0.003, 0.001	0.23, 0.25
2-DOG	1.57, 1.48	0.23, 0.18
Glucose	3.24, 3.05	0.35, 0.41
Glucose plus 2-DOG	3.98, 5.60	0.14, 0.26
Glycerol	1.94, 1.94	0.32, 0.20
Glycerol plus 2-DOG	4.17, 3.51	0.08, 0.23

Table 4. Effect of insulin and actinomycin on the depression of liver phosphoenolpyruvate carboxykinase in rats made diabetic by alloxan. A single subcutaneous injection of 20 units of protamine zinc insulin was given in the late afternoon. At 0 and 6 hours, actinomycin (175 units) was administered intraperitoneally. The animals were killed after 12 hours. The initial glucose concentration in the blood of all diabetic rats exceeded 440 mg/100 ml. Numbers in parentheses represent number of rats.

Compound administered	PEP carboxykinase (nmole · min^{-1} · mg of $protein^{-1}$)
None (5)	217 ± 21
Insulin (5)	61 ± 15
Insulin plus fasting for 12 hours (2)	208
Actinomycin (5)	144 ± 12
Actinomycin plus insulin (5)	90 ± 18

to inhibit the deposition in muscle. These findings can probably be explained best by (i) the inhibition by 2-DOG of glucose transport in muscle (*15*) (by contrast, glucose penetration into liver cells is not dependent on a transport mechanism); and (ii) inhibition by 2-DOG-6-phosphate of hepatic glucose 6-phosphatase (*12*) with consequent diversion of glucose monophosphate from degradation to glycogen formation and accumulation. Two explanations may be offered for the observation that 2-DOG did not inhibit the (presumed) conversion of glycerol to liver glycogen. Liver phosphohexoisomerase may be less susceptible to inhibition by 2-DOG-6-phosphate than the corresponding enzyme in other tissues (*12*), or the concentration of the inhibitory 2-DOG-6-phosphate may be kept low by the activity of glucose 6-phosphatase. We have interpreted these results to indicate that the influence of administered carbohydrate on hepatic PEP carboxykinase may be exerted in tissues other than liver.

Insulin is effective in depressing enzymes related to gluconeogenesis, but the question of its primary action at the gene level remains controversial (*16*). Table 4 presents data which show that insulin depresses PEP carboxykinase activity only when a dietary source of carbohydrate is available. Insulin did not depress the enzyme activity in fasting, diabetic rats. An attempt was made to determine whether insulin could depress PEP carboxykinase activity in the face of blocked RNA and protein synthesis. In some experiments (Table 4), insulin and actinomycin were given alone, and in combination, to diabetic rats with increased PEP carboxykinase activity. There is some indication that actinomycin plus insulin produced less depression than insulin alone, but the fact that actinomycin alone caused significant depression renders this type of experiment inconclusive.

Our studies suggest the possibility that some site other than liver may initiate the effect of glucose on PEP carboxykinase. A humoral factor or metabolite picked up by the liver from the circulation could, in turn, suppress gluconeogenesis. Even more likely, the concentration of circulating amino acids could determine the activity of PEP carboxykinase. Lack of circulating glucose or lack of glucose utilization (in diabetes or when 2-DOG was given) would cause amino acids to be liberated from peripheral tissues when adrenals are intact (*17*); administered gluconeogenic amino acids cause increased activity of PEP carboxykinase even in fasted rats (*4*). Conversely, available glucose would diminish this stimulus to enzyme formation. While our studies do not contradict the many diverse effects produced by insulin, they are consistent with the unitary hypothesis that this hormone works at the cell membrane of responsive tissues to regulate glucose utilization (*18*).

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A Requirement for Two Cell Types for Antibody Formation in vitro

Abstract. *A suspension of mouse spleen cells can be separated into two populations on the basis of their ability or inability to adhere to plastic dishes. It was found that both adherent and nonadherent cells were necessary for the induction of antibody formation to sheep red blood cells in vitro. Exposure of adherent cells to antigen for brief periods of time was sufficient to initiate a maximal in vitro response.*

Antibody synthesis may result from the interaction of two functionally different cell types: one which phagocytizes and "processes" the antigen to provide the stimulus for a second type, the lymphoid cells which synthesize specific antibody (*1*). Unequivocal support for this suggestion has not been obtained from in vivo experiments. The following studies, however, in which mouse spleen cells are cultured in vitro with sheep erythrocytes as antigen after the method of Mishell and Dutton (*2*), provide provisional support for the hypothesis that two functionally different cell types indeed are required for the induction of antibody synthesis to the antigen.

Spleen cell suspensions were prepared by gently teasing apart the spleens of unimmunized DBA/2 Jax mice in cold

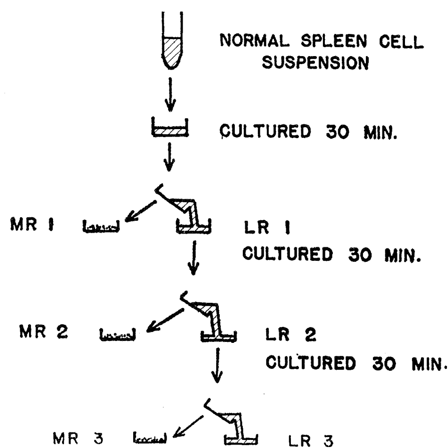

Fig. 1. Method of culturing DBA mouse spleen cells to obtain adherent (M.R.) and nonadherent (L.R.) populations.

Hanks's balanced salt solution. Cell aggregates were allowed to sediment briefly and the resulting supernatant was centrifuged 10 minutes at 600*g* at 4°C. The pooled sedimented cells were resuspended to a concentration of 10^7 cells/ml in Eagle's minimum essential medium supplemented with nonessential amino acids, pyruvate, glutamine, and 10 percent fetal bovine serum. Each culture contained 1 ml of the cell suspension in a 35- by 10-mm plastic culture dish (Falcon). Cultures were maintained on a tilt table (Bellco, Vineland, N.J.) at 13 oscillations per minute in an atmosphere of 7 percent O_2, 10 percent CO_2, and 83 percent N_2 as described by Mishell and Dutton (*2*). To each dish were added daily 0.05 ml of twice concentrated medium and 0.05 ml of fetal bovine serum.

Table 1. Three separate experiments illustrating the plaque-forming-cell response in vitro of various cell populations. In each experiment the populations were derived from a pool of DBA mouse spleen cells and exposed to sheep red blood cells (SRBC) in vitro. Values are numbers of plaque-forming cells per 10^6 cells at initiation of culture.

Cell populations	Day-4 response		
	Expt. 1	Expt. 2	Expt. 3
Normal spleen cells	1	2	1
Normal spleen cells + SRBC*	170	60	342
(MR 1 + SRBC) + normal cells	215	96	280
(MR 2 + SRBC) + normal cells	115	45	172
(MR 3 + SRBC) + normal cells	90	36	96
(MR 1 + SRBC) + LR 3	50	20	110
(MR 2 + SRBC) + LR 3	125	30	105
(MR 3 + SRBC) + LR 3	50	50	70
MR 1 + SRBC†	1	0	0
MR 2 + SRBC†	0	0	0
MR 3 + SRBC†	0	0	0
LR 1 + SRBC	6	2	4
LR 2 + SRBC	0	0	0
LR 3 + SRBC	0	0	0

* 10^7 sheep red blood cells. † Exposure to antigen either 30 minutes or throughout culture gave the same results.

Normal spleen cells were separated into two populations: one population which adheres to plastic during a 30-minute culture interval, and a second population which does not adhere to plastic during the same interval. Thus, separation of cells was based on a functional property, adherence or nonadherence to plastic during a short interval of culture. Ten million spleen cells per culture dish were cultured for 30 minutes and the nonadhering cells were removed by aspiration and used for the preparation of further adherent and nonadherent cell populations. About half of the cells initially plated remained in the culture dish. These remaining cells were either firmly or loosely adherent to the plastic. The loosely adherent cells were removed by three gentle washings with balanced salt solution and discarded. About one million cells remained firmly attached to the dish; most of these cells rapidly phagocytized titanium dioxide and for convenience are referred to as a "macrophage rich" (M.R.) population (*3*). Culture of the nonadherent cells for two more intervals of 30 minutes each removed additional cells capable of adhering to plastic. Most of the nonadherent cells had the morphological characteristics of small lymphocytes and for convenience will be termed a "lymphocyte rich" (L.R.) population. This method of cell separation is shown diagrammatically in Fig. 1. Cells adhering to plastic after each of the three periods of culture are referred to as M.R. 1, M.R. 2, and M.R. 3 populations. Corresponding nonadherent cells are referred to as L.R. 1, L.R. 2, and L.R. 3 populations.

The number of cultured cells releasing antibody to sheep red blood cells was enumerated by a modification of the Jerne technique with microscope slides. Cultured cells, including cells free in the culture medium and cells adherent to plastic, were harvested by scraping the dishes with a plastic policeman. The harvested cells were washed, dispersed by agitation with a vortex mixer, and added to agarose containing sheep red blood cells (*4*). Results are

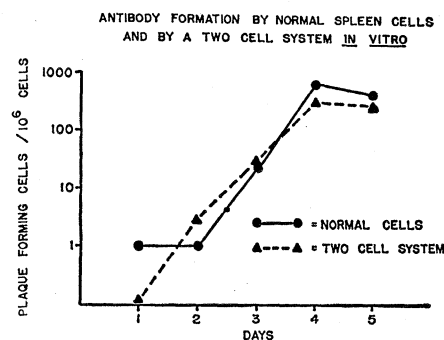

Fig. 2. The plaque-forming-cell response in vitro of normal DBA mouse spleen cells and M.R./L.R. cells cultured with 10^7 sheep red blood cells. Antigen was given only to the M.R. cells in the two-cell system (see text).

expressed as plaque-forming cells per 10^6 cells initially cultured. In repeated experiments the optimal dose of antigen was 10^7 sheep red blood cells per culture dish; increasing the dose of antigen tenfold did not enhance the response further, and decreasing the dose to 10^6 red blood cells produced significantly fewer plaque-forming cells. An exponential increase in the number of antibody-releasing cells occurred between day 2 and day 4 when 10^7 normal spleen cells were cultured with 10^7 sheep red blood cells, as indicated by the solid line in Fig. 2. An identical response was obtained when L.R. 3 cells were added to M.R. 1 cells (see Fig. 1) and the recombined cell populations were cultured with 10^7 sheep red blood cells. Thus, the procedure for separating the two cell populations had not impaired the capacity of the recombined cells to respond to antigen. No plaque-forming-cell response occurred if either M.R. 1 (1×10^6 cells per dish) or L.R. 3 (adjusted to 10^7 cells per dish) cells were cultured with 10^7 sheep red blood cells, indicating that both cell types were essential for the response.

The following experiment demonstrated that antigen rapidly phagocytized by M.R. cells could stimulate the response of the L.R. cells. Macrophage-rich cells were cultured with 10^7 sheep red blood cells for 30 minutes. The culture fluid was aspirated and each culture dish was washed three times with balanced salt solution to remove erythrocytes not phagocytized. Examination of the dishes by phase microscopy showed sheep red blood cells present within some macrophages but no free sheep red blood cells. An estimated 5 percent or less of the antigen dose had been phagocytized in 30 minutes

(5). Lymphocyte-rich cells (10^7) were added to each culture dish containing 10^6 M.R. cells and phagocytized sheep red blood cells. The plaque-forming-cell response was equivalent to that of normal spleen cells with 10^7 sheep red blood cells present throughout culture, and is indicated by the broken line in Fig. 2. These results were confirmed in subsequent experiments. A single pool of normal spleen cells was divided into various populations as listed in Table 1. A M.R. population (M.R. 1, 10^6 cells per dish; M.R. 2, 5×10^5 cells per dish; M.R. 3, 2.5×10^5 cells per dish) was incubated with 10^7 sheep red blood cells per culture dish for 30 minutes. The dishes were washed carefully, and either 10^7 normal spleen cells or 10^7 L.R. 3 cells were added to each culture dish. Both methods of culture produced a significant plaque-forming-cell response, comparable in magnitude to that produced by normal spleen cells continuously exposed to antigen. Little or no response occurred if either M.R. cells or L.R. cells alone were cultured with 10^7 sheep red blood cells; the very small response of the L.R. 1 cells can be attributed to a few macrophages remaining in this cell population. Similar results were obtained in two additional experiments of identical design presented in Table 1.

Thus, both adherent and nonadherent mouse spleen cells are essential for development of plaque-forming cells in vitro. Moreover, an amount of phagocytized antigen estimated to account for less than 5 percent of the total antigen dose is sufficient to produce a maximum in vitro immune response. That more antigen is not required for immune induction is consistent with the studies of several workers (6).

It appears that in the mouse spleen, production of antibody to sheep erythrocytes involves both antigen phagocytosis by macrophages and macrophage lymphocyte interaction, both processes being essential for development of lymphoid cells releasing hemolytic antibody. It has been suggested that transfer of information between two cell types involves RNA or RNA-antigen complexes (7). It may now be possible to determine the nature of "information transfer" between two cell types by using in vitro induction of antibody synthesis.

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3. Macrophage-rich cells were not enumerated in each experiment reported, but in a separate set of experiments the adherent cells were eluted by culturing for 30 minutes in medium supplemented with 30 m*M* ethylenediaminetetraacetate. The M.R. 1 population was found to contain 1×10^6 cells per culture dish, the M.R. 2 population 5×10^5 cells per dish, and the M.R. 3 population 2.5×10^5 cells per dish. Approximately 95 percent of adherent cells actively phagocytized sufficient titanium dioxide (0.01 percent weight/vol) during 30 minutes of culture to contain many refractile granules visible by phase microscopy.
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5. Ten replicate hemacytometer counts were made on (i) the suspension of sheep red blood cells used to inoculate the culture dishes and (ii) the suspension of sheep red blood cells obtained by combining the recovered culture fluid and the repeated washings after the 30-minute culture interval. By this method of enumeration, as many sheep red blood cells were recovered as were inoculated. Considering the error of replicate counts to be 5 percent or less with the dilution method employed, 5×10^5 or fewer sheep red blood cells are estimated to have been phagocytized by the macrophages.
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Growth of Isolated Mesophyll Cells of Arachis hypogaea in Simple Defined Medium in vitro

Abstract. *Isolated mesophyll cells from leaflets of* Arachis hypogaea *can be cultured in a simple, defined liquid nutrient medium containing minerals, with an appropriate source of ammonia, sugar, 2,4-dichlorophenoxyacetic acid, and kinetin. The significance of such a simple medium in understanding the problems of cell metabolism, growth, and morphogenesis is discussed.*

In recent years much success has been achieved in the isolation and culture of single cells of higher plants from callus cultures (1). In most investigations, complex growth substances such as coconut milk, yeast extract, and other substances were used as supplements to the final media, and thus the understanding of specific growth requirements of the isolated cells and of cell morphogenesis is restricted. Attempts to obtain a defined medium for the growth of such cells (2) showed the exogenous requirement for various metabolites, which only reveals the heterotrophic potential of the cells. The understanding of plant morphogenesis ultimately depends upon the isolation of a strain of cells that is completely autotrophic.

According to Steward *et al.* (3), if cells are to exhibit their full totipotency, two requirements must be met: (i) a cell must be freed from organic connections with other cells, and (ii) free cells must be nourished by a medium which is fully competent to support their rapid growth and development. It has been widely accepted that formation of embryo-like structures from single carrot cells requires the presence of coconut milk (4). However, Halperin (5) has shown that embryogenesis occurs readily in cultures of wild carrot on media containing only minerals, sucrose, vitamins, and an

Table 1. Effect of added ammonium citrate, tartrate, or nitrate on the growth and chlorophyll (Ca and Cb) formation of isolated mesophyll cells of *Arachis hypogaea* in basal medium.

Ammonium salts added (mg)	Final fresh wt. (mg)	Growth value	Chlorophyll content (μg/g fresh wt.)	
			Ca	Cb
		Citrate		
None	183.3	305.5	109.7	49.1
400	382.1	636.8		
800	189.7	316.1	53.3	24.7
1600	Insignificant growth, few pale green spheres formed			
		Tartrate		
400	342.2	570.3	36.6	20.9
800	335.3	558.8	60.2	26.2
1600	210.2	350.3	43.6	30.9
		Nitrate		
400	120.0	200.0	115.2	74.8
800	224.0	373.3	117.5	64.2
1600	51.2	85.3	105.9	57.1