Plaque Forming Cells Produced in Test Tube Cultures of Normal Mouse Spleen Cells Incubated with Erythrocytes[1] (36404)

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The immunological events leading to the synthesis of antibody are still largely unknown, and recent studies have even raised the question of participation by more than one kind of cell in the synthesis of an antibody (1), whether by direct contact between such cells (2, 3) or by passage of some substance from one cell to the other (4, 5). Attempts to clarify these processes have been largely by the transfer of cells to X-irradiated animals (6), and, more recently, by a new method for *in vitro* culture (7) in which antibody synthesis was induced either as a primary (8) or secondary immune response.

In the course of current studies on blastogenesis in cultures of mouse spleen cells, a method was developed for inducing an immune response *in vitro* in spleen cells of normal mice by a procedure substantially simpler than that referred to above: a test tube culture in which normal mouse spleen cells, after settling with antigenic RBC, can produce plaque forming cells, provided the cells are not disturbed during the incubation. This system, and some of the data obtained in immune responses to sheep or burro red blood cells, are presented below.

Materials and Methods. BALB/c female mice, 8–12 weeks old, were obtained from The Jackson Laboratory, Bar Harbor, ME. Spleens were aseptically removed and teased, and the cells were washed and suspended in RPMI-1640 medium supplemented with 10% heat-inactivated IPT fetal calf serum (IPT FCS, Grand Island Biological Co., Grand Island, NY 14072, Cat. No. 651).

The spleen cell suspension, 10^6/ml, was distributed in plastic tissue culture tubes (Falcon Plastic, Cat. No. 3033), at 3 ml/tube. Sheep red blood cells (SRBC) were obtained from Baltimore Biological Laboratory, (Cockeysville, MD 21030) and burro red blood cells (BRBC) from Davis Laboratories (P.O. Box 489, Davis, CA) both in Alsever's solution. The RBC were washed 3 × in RPMI-1640 medium and suspended at 1% (approx 10^8/ml), of which 0.1 ml was added to the tubes at the initiation of the culture. The tubes were incubated for 30–60 min at 37° in a multipurpose rotator Model 150 V (Scientific Instruments, Inc., Springfield, MA) at its lowest speed, and then placed vertically in a 37° humidified CO_2 incubator containing 95% air and 5% CO_2. After 3 days, 1.0 ml of fresh RPMI-1640 medium without serum was added, without disturbing the button of cells which had formed. After 6 days (unless otherwise noted) the cultures were harvested by centrifuging the tubes, decanting the supernatant, shaking the tubes gently, and resuspending the cell pellet in 0.6 ml of medium for determination of the number of cells producing antibody to SRBC by the hemolytic antibody plaque technique of Jerne and Nordin (9). The cells were plated by adding to each tube 1 ml of 1.2% agarose (L'Industrie Biologique Francaise, S. A. Gennevilliers, Seine, France) in Earle's solution at 47°, with 3% SRBC or BRBC, mixing, and spreading 1 ml on a prepared layer of hardened agarose in 60 mm petri plates (Falcon Plastics, Los Angeles, CA 90045, Cat. No. 1007). After 60 min of incubation at 37° under 1 ml of 1:20 normal

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TABLE I. PFC/10^6 Normal BALB/c Mouse Spleen Cells Cultured with Burro RBC.[a]

No. of spleen cells $\times 10^6$	No. of BRBC	PFC/10^6 cells
3	10^8	39
3	10^7	102
3	10^6	49
3	2×10^5	35
6	10^8	35
6	10^7	26
6	10^6	14
6	2×10^5	39
9	10^8	19
9	10^7	32
9	10^6	43
9	2×10^5	18
12	10^7	20
15	10^7	11

[a] Control, without red cells, 5–15. All results are the means of duplicate cultures, harvested after 6 days.

guinea pig serum, plaques were counted. Cell viability was determined by trypan blue exclusion.

Results and Discussion. The number of spleen cells which excluded trypan blue after 6 days of culture varied between 20 to 30% of the total number of cells initially seeded. It was found that immediately after teasing, the viability fell to approximately 75% of the total population, but there was no attempt to compensate for this loss of viable cells. Thus, all results given are per 10^6 cells initially cultured.

In preliminary experiments, cultures were set up to define the optimal concentration of FCS for supporting the culture, and concentrations of 0, 5, 10, 15% were compared. Antibody producing cells developed in the culture were enumerated by their ability to produce plaques of hemolysis in agar against the RBC used as the antigen (9). IPT FCS at 10% yielded the highest number of plaque forming cells (PFC). In other experiments the ratio of spleen cells to RBC was studied and 10^7 SRBC were found to be optimal for culturing with 3×10^6 spleen cells (Table I). While the conditions for the initiation of the response were being defined, a number of experiments were set up to determine the level of the response in relation to the period of culture. Table II shows an increase from day 4 to a level of 60–70 PFC/10^6 on day 5 and then a continued increase, at a lower rate, until day 7, when the level of PFC/10^6 reached 97–110, with a sharp decrease thereafter. The plaque counts of control cultures, incubated without antigen, showed little change from the level of 18–22 after day 5. As was found by Mishell and Dutton (8), burro RBC gave a substantially lower background of plaque counts, but generally induced a lower level of response than did SRBC.

Note that occasionally an entire set of cultures would fail to produce PFC, or would yield very low numbers, in the range of 10–20% of the expected range. When such failures occurred it was most often in a series of two and even three consecutive experiments. In these series the same batch of sheep or burro blood had been used.

The method described above has been found satisfactory for initiation of an immune response to SRBC and BRBC *in vitro*. In contrast to other methods of achieving this (3, 8), rocking was not found essential for eliciting the response, except for the rolling of the tubes after addition of the RBC at the initiation of the culture. The essential condition for this method would appear to be the creation and maintenance of a microenvironment in which the stimulated lymphocytes can function, if the button of cells is not disturbed. Differences from methods previously described, in cell density, frequency of feeding, and optimal time of response, may be related to this mechanism. The rise in number of plaque forming cells between days 4 to 6 clearly indicates the development of antibody producing cells during this period. It should be pointed out that the cells are quite fragile at the end of this period in culture, so that more vigorous washing than that suggested for plating here can cause a substantial decrease in the number of plaques obtained.

In an assessment of this method, in comparison to that of Mishell and Dutton (8), it should be pointed out that the PFC/10^6 cells reported here are relative to the starting

TABLE II. PFC/10^6 Normal BALB Mouse Spleen Cells Cultured for Various Periods with Red Blood Cells of Sheep or Burro.[a]

Expt.	Days in culture									
	4		5		6		7		8	
No.	RBC	Control	RBC	Control	RBC	Control	RBC	Control	RBC	Control
SRBC TC EG 1	6	1	74	8						
TC EG 2	10	4	78	7						
TC 6-11			69	18	90	24	110	14		
TC 13			75	21	84	22				
TC 71			73	22	84	20	97	15	38	12
TC EG 3					82	18				
BRBC TC 9-16			75	11	93	9				
TC EG 4					81	12				
TG EG 5					50	5				

[a] All tubes contained 3×10^6 spleen cells, and all contained 10^7 RBC except the control tubes.

number of cells cultured. Since our level of viability was about 30%, the PFC per million viable cells at time of harvest would be obtained by multiplying the PFC/10^6 cells given here by a factor of 3–4. The level of 200–300 PFC/10^6 viable harvested cells which would thus be obtained from our data is lower than that achieved by Mishell and Dutton (8), probably because of better biochemical conditions achieved in their cultures. However, the level attained in the cultures described here is well within the range which can be used for studying factors in antibody formation, and the simplicity of this method may widen the range of experiments which can be done in this area. The apparent importance of the microenvironment for the cultured cells suggests an essential similarity to the method of Marbrook (10).

Summary. Test tube cultures of normal mouse spleen cells set up as for mixed leukocyte culture, but with antigenic RBC, and fed once with additional medium without disturbing the button of cells, were found to contain antibody producing cells, as indicated by the ability of such cells to produce hemolytic antibody plaques when plated against the RBC. In the case of sheep RBC as antigen, the number of PFC increased to day 7, when a level of 90–110 per 10^6 starting spleen cells was reached, in comparison with 18–24 PFC in control cultures, without antigen. With burro RBC such results were also obtained, with somewhat lower levels of PFC produced in antigen-containing cultures, but substantially lower background levels. Since dispersion of the cells at re-feeding caused failure of appearance of the PFC, a condition for the production of these was presumably the formation of a microenvironment within the button of cells in the bottom of the tube.

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