MATURATION OF HEMOLYSIN-PRODUCING CELL CLONES

I. The Kinetics of the Induction Period of an In Vitro Hemolysin Response to Erythrocyte Antigen*

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The events which occur during the induction period of an immune response remain obscure despite much investigation. The recent report of Marbrook (1) provides a system in which precursor cell units capable of interacting with erythrocyte antigens to produce hemolysin-producing cell clones (HPCC) may be directly studied. The in vivo methods (2–4) in which precursor cell units may be detected and enumerated do not allow direct study of the induction period. Other in vitro systems (5–10) for studying antibody synthesis measure only the cells (or their products) which result from interaction of precursor units with antigen and do not allow for the study of the precursor units themselves. The experiments to be reported here indicate that precursors of plaque-forming cells (PPFC) maturate to the point of antibody synthesis, probably in the absence of cell division, within 4 hr after antigenic stimulation. Following initial maturation there is a lag period of from 12–13 hr before replication of hemolysin producing progeny begins. In the reported system, cell division, once initiated, proceeds synchronously for at least the first three cell doublings, with a generation time of from 7–8 hr.

Materials and Methods

Mice.—6–10 wk-old male CBA mice obtained from Jackson Laboratories, Bar Harbor, Maine, were used in all experiments.

Culture Reagents.—The following reagents were obtained from Hyland Laboratories, Los Angeles, Calif.: Hanks' balanced salt solution without bicarbonate (HBSS), 10X concentration (065-355); Eagle's minimum essential medium (MEM), Hanks' base, IX concentration (065–517); MEM essential amino acids, 50X concentration (065-500); MEM nonessential amino acids, 100X concentration (065-540); L-glutamine 5% dried (65-656); sodium pyruvate, 100X concentration (65-550); MEM vitamins, 100X concentration (065-510). Fetal calf serum (FCS) was obtained from Reheis Chemical Company, Chicago, Ill. (Rehatuin FS lot No. E-19701). IX stock medium was prepared by adding 1.0 ml MEM nonessential amino acids, 1.0 ml sodium pyruvate, 0.6 ml L-glutamine, 10.0 ml FCS, and 10,000 units penicillin G to 100

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ml MEM. Completed medium was stored at −20°C in 10 ml aliquots. Freezing had no adverse effects on the medium so long as sodium bicarbonate was not added until stored medium was thawed for use. At this time 0.15 ml of 7.5% $NaHCO_3$ was added per 10.0 ml stock IX medium. (If $NaHCO_3$ is added to the medium prior to freezing, insoluble precipitates form on thawing.) 2X stock medium was prepared by adding 2.0 ml MEM essential amino acids; 2.0 ml MEM nonessential amino acids; 2.0 ml sodium pyruvate; 1.0 ml MEM vitamins; 1.2 ml L-glutamine; 20.0 ml FCS; 11.0 ml 10X HBSS; and 20,000 units penicillin G to 100 ml MEM. Completed 2X stock medium was stored at −20°C in 10.0 ml aliquots. After thawing for use 0.3 ml 7.5% $NaHCO_3$ was added per 10 ml of 2X medium.

Antigen.—Sheep (CS1111), chicken (CS1151), or horse (CS0003) erythrocytes obtained from Colorado Serum Company, Denver, Colorado, were washed three times in HBSS. A 5% suspension of washed cells was prepared by adding the appropriate volume of 1X stock medium to the packed cells obtained after the final wash.

Cell Culture.—The technique of Marbrook (1) with slight modifications was used throughout these experiments. The lower solid phase was prepared by combining and mixing equal volumes of warmed (48°C) complete 2X stock medium and 0.7% agarose (48°C) (Mann Research Laboratory, New York). 2.5 ml of this mixture was added to individual 60 mm sterile disposable plastic Petri dishes (Falcon Plastics, Los Angeles, Calif., No. 1007). The upper solid phase was likewise prepared except that 0.1 ml of the appropriate erythrocyte suspension was added per 1.0 ml agarose-medium mixture. After the lower phase had solidified 1.0 ml of the upper solid phase was added to each Petri dish and allowed to harden. Agarose was used for these experiments because: (*a*) The need for DEAE-dextran in solid phases is eliminated; other workers (11) have shown DEAE-dextran to inhibit the expression of plaque-forming cells (PFC). (*b*) It was found that less variation in results occurred both among replicate cultures within one experiment and from experiment to experiment when agarose was used in the solid phases instead of agar. The liquid phase consisted of 1.0 ml 1X stock medium to which the appropriate number (usually about 10^7) of dissociated mouse spleen cells was added. Additional antigen was not added to the liquid phase. More than enough antigenic determinants are present on the surface of the upper solid phase to initiate an antibody response. Cultures were incubated at 37°C in a humidified atmosphere containing 7% O_2, 10% CO_2, and 83% N_2. Cultures were neither rocked nor fed during the course of the incubation period. At appropriate times cultures were harvested and analyzed both for precursor units and for total PFC.

Precursor Units and PFC.—A precursor unit responsible for the development of a clone of hemolysin producing cells can be defined as the sum total of all cells and their interactions which in the presence of antigen induce the formation of a clone of hemolysin producing cells. Precursor units per culture were enumerated as follows. The liquid phase of each culture containing mouse spleen cells was aseptically pipetted (and saved) from the Petri dish. The surface of the upper solid phase was washed once with HBSS and the wash was added to the tube containing the liquid phase. (The liquid phase was assayed for total PFC as will be described shortly.) 1.0 ml of guinea pig serum as a source of complement (Texas Biological Laboratories, Fort Worth, Texas) diluted 1:10 in HBSS was added to each washed Petri dish and incubated for 90 min at 37°C. The areas of lysis which developed on each dish were counted and recorded. Each area of lysis which developed theoretically resulted from the maturation of a precursor unit into a clone of hemolysin producing cells. Total PFC per culture were determined by the method of Jerne and Nordin (12). Harvested spleen cells were centrifuged at 400 rpm for 3 min. The supernatant was discarded and 0.1 ml of the appropriate erythrocyte suspension added. The cell pellet was thoroughly dispersed. 60 mm plastic Petri dishes were utilized for the assay. The bottom layer consisted of 3.0 ml of 0.7% agarose-2XHBSS mixture. The top layer contained 0.9 ml of 0.7% agarose-2X stock medium mixture plus 0.1 ml erythrocyte suspension containing the harvested spleen cells from a single culture. The plates were incubated for 6 hr at 37°C in a humidified atmosphere containing 7% O_2, 10% CO_2, and 83% N_2.

1.0 ml of 10% guinea pig serum was then added to each dish followed by an additional 90 min incubation in air at 37°C. The plates were stained with Benzidine, coded, and counted by at least two individuals. Plaques from a single plate were expressed as PFC/culture. The relatively long (6 hr) initial incubation in the presence of medium was found to be necessary for maximum expression of PFC. This may be explained by the fact that during their development in culture PFC are in constant contact with antigen and it would appear logical therefore that this antigen acts like a sponge to draw antibody from the cell surface of producing cells as soon as it is released. In addition, during and shortly after cell division the rate of antibody synthesis is probably quite low (13). In order to insure maximum PFC survival it was also found very important to keep the agarose-medium-cell suspension mixture at no more than 48°C and to pour this mixture onto the Petri dish as quickly as possible.

Metabolic Inhibitors.—Puromycin dihydrochloride, control No. 8257 was obtained from Nutritional Biochemical Co., Cleveland, Ohio. 5-fluoro-2-deoxyuridine (FUdR) was obtained from Hoffman-LaRoche, Inc., Nutley, N. J.

A unique property of this experimental system is that for all practical purposes all cells capable of responding to antigen actually contact antigen at about the same time. Spleen cells rapidly settle onto the upper solid phase which contains approximately 10^8 SRBC of which (by calculation) at least 10^7 RBC have at least a portion of their cell surface exposed at the surface. This property possibly explains the synchrony observed in these experiments. In the in vivo situation and in the Mishell and Dutton (9) in vitro system such synchrony is not observed (unpublished observations), possibly due to the fact that antigen does not simultaneously contact all cells capable of specifically reacting to it.

Perhaps the most important aspect of the technique is the care in which Jerne assays must be prepared. To accurately do the kinetics experiments reported here, one must have an assay system which very efficiently determines the number of PFC per culture. To obtain this efficiency, lumpy, unevenly poured plates must be avoided. Plates should be coded and hand counted in indirect light by at least two people. In our experience counting the plates by using an overhead projector consistently gives a total count of 10–20% less plaques than does hand counting.

PRELIMINARY OBSERVATIONS

Kinetics.—The kinetics of developing hemolysin producing cell clones (HPCC) in culture is illustrated in Fig. 1. In this typical experiment using sheep erythrocyte antigen, it can be seen that the number of precursor units does not significantly change from 24–96 hr. However, the total number of PFC does increase during this same period; for about 48 hr after antigenic stimulation this increase is exponential. After 48 hr the curve flattens out; possible causes for the loss of exponential growth will be discussed later. During the period of exponential growth, the doubling time of PFC was calculated to be 7.2 hr. The observation that the number of precursor units/culture remains stable throughout a 4 day experiment indicates that recruitment of additional cells capable of producing clones of PFC does not occur in this system. This data is in agreement with the recent findings of Rowley et al. (13).

Controls.—Direct negative controls in a system of this type are not possible. However the following indirect controls were done and their results are summarized in Table I. Spleen cell suspensions cultured in the absence of any erythrocyte antigen yielded few PFC upon assay against sheep, chicken, or

horse erythrocytes. Spleen cell suspensions incubated in the presence of erythrocyte antigens yield large numbers of PFC when assayed against the specific antigen present in the culture but yielded much fewer PFC when assayed

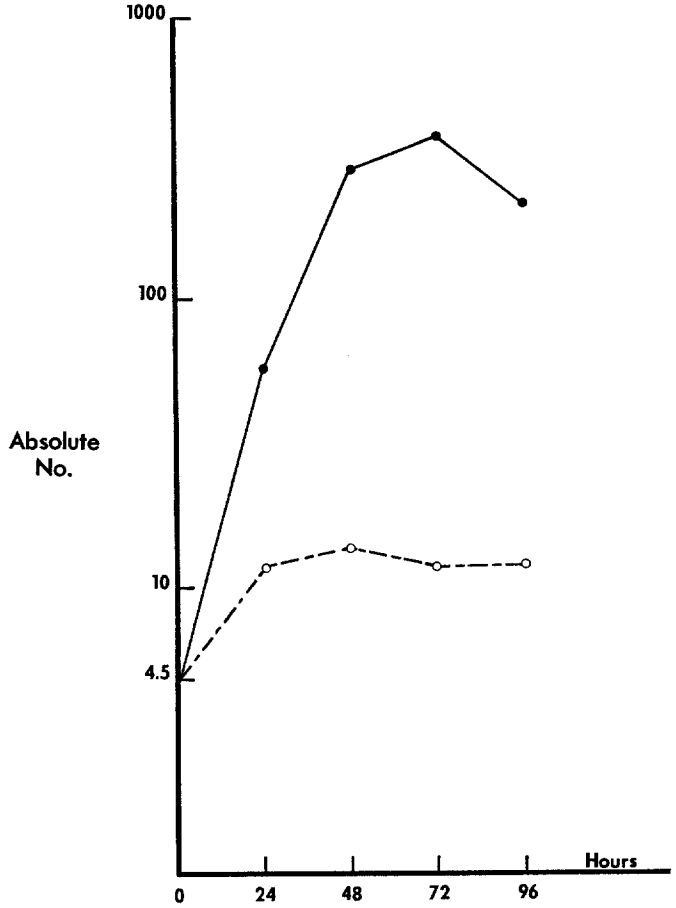

FIG. 1. Time relationship of the appearance of total PFC/culture with respect to the number of plaques on the culture dish (precursor units) in a typical Marbrook experiment. (1×10^7 mouse spleen cells/culture incubated in the presence of SRBC.) Each point on each curve represents the mean of four cultures. O, precursor units/culture; ●, total PFC/culture.

against other erythrocyte antigens. Therefore the PFC appear to result from a specific interaction of precursor units with antigen.

I would like to stress the importance that the "lot" of FCS to be used in this type of system should be carefully tested for the presence of cross-reacting antigens against the erythrocytes utilized. Some lots of FCS (especially non-

filtered sera) contain large amounts of antigenic substances capable of inducing (in the absence of any added erythrocyte antigen) the proliferation of cells capable of complement-dependent lysis of sheep and other erythrocytes. Naturally one should take care to use FCS in which the presence of these cross-reacting antigens is either absent or, at most, minimally present. In the experiments reported here the FCS used did not contain significant amounts of cross-reacting antigens.

RESULTS

Detailed Kinetics.—The kinetics of the appearance of plaques on the surface of the culture dish (indicating the presence of precursor units) and the paral-

TABLE I

Specificity of the PFC Response in the Marbrook System

Erythrocyte antigen in culture	48 hr response		
	PFC/culture* assayed against		
	Sheep	Horse	Chicken
None	7.8	8.3	9.0
Sheep	230	27	38
Horse	31	194	26
Chicken	17	9.5	246

* Mean of at least four cultures—1×10^7 spleen cells initially seeded per culture.

lel development of PFC was studied in detail in the following four experiments using sheep erythrocyte antigen (SRBC). In these experiments cultures were harvested at intervals after exposure to antigen and assayed for both precursor units and PFC.

In the first experiment (0–8 hr), summarized in Table II, the number of plaques on the culture dish reaches a maximum at 4 hr, thus indicating that precursors of plaque-forming cells (PPFC) rapidly maturate to the point of hemolysin production. In subsequent points the size of the plaques does increase, however. During this entire period the total PFC/culture remains approximately equal to the number of precursor units/culture, thus indicating that replication of hemolysin forming progeny has not yet begun.

In the second experiment (Table III) cultures were harvested and assayed at 2 hr intervals from 11–23 hr after exposure to antigen. The number of precursor units/culture remains stable during this period but between 17 and 19 hr the total PFC/culture have apparently doubled, suggesting a synchronous division (Fig. 2). From 19–23 hr total PFC/culture do not significantly change.

In the next two experiments (Table IV) from 22–32 hr and 18–32 hr after

antigen, the number of precursor units again does not change during the course of the observations. In each experiment two synchronous doublings of PFC has occurred (Fig. 3). The time between doublings is 7–8 hr.

The initial synchronous doubling of hemolysin-forming cells in these experiments strongly implies that PPFC are all at the same point in the cell cycle when they are initially stimulated. It also follows that all PPFC in this system are stimulated almost simultaneously.

Effects of Metabolic Inhibitors

Puromycin.—To ascertain whether initial maturation of PPFC is dependent on the synthesis of new protein, the following experiment was done. Spleen

TABLE II

Kinetics of Developing HPCC (0–8 hr)

Time	Plaques in* culture dish‡	PFC* / culture
hr		
0	—	4.5§
2	4	3
3	5	5
4	13	10
6	12.5	13
8	11	8

* Mean of four cultures—1 × 10^7 spleen cells initially cultured.

‡ To be called *precursor units* in subsequent tables.

§ Mean of four Jerne plates. 1 × 10^7 spleen cells/plate incubated at 37°C for 6 hr in a humidified atmosphere containing 83% N_2, 7% O_2, 10% CO_2. The plates contained only agarose and HBSS; no growth medium was supplied.

cells were incubated for 1 hr at 37°C in IX gassed stock medium containing various concentrations of puromycin dihydrochloride, but no antigen. At the end of this hour the spleen cells and medium were placed on culture dishes containing antigen (SRBC) whose solid phases also contained puromycin at the same concentration as the respective liquid medium. After 48 hr of incubation the cultures were assayed for both precursor units and PFC. The results are summarized in Table V and indicate that the appearance of the initial plaques in the culture dish are dependent on the synthesis of new protein. Cell viability, as determined by trypan blue exclusion, was not significantly affected within the 48 hr incubation period by puromycin concentrations of up to 12.5 μg/ml. However, concentrations of 25 and 50 μg/ml were directly toxic to the cultured spleen cells. Concentrations of puromycin as low as 1 μg/ml appeared to interfere with replication of PFC.

FUdR.—To determine whether maturation of PPFC depends on synthesis

of new DNA, spleen cells were cultured with various concentrations of FUdR in the presence of SRBC. After 48 hr of incubation, cultures were harvested and assayed for precursor units and PFC (Table VI). The results indicate that the initial maturation of PPFC to the point of hemolysin production does not

TABLE III

Kinetics of Developing HPCC (11–23 hr)

Time	Precursor* units / culture	PFC* / culture	PFC / pre. unit	Approximate number of doublings
hr				
11	9.0	7.5	0.84 ± .26‡	0
13	10.5	8.0	0.76 ± .21	0
15	11.0	11.8	1.07 ± .25	0
17	9.8	7.0	0.72 ± .43	0
19	10.0	20.0	2.00 ± .21	1
21	10.0	17.0	1.70 ± .17	1
23	11.8	25.2	2.14 ± .22	1

* Mean of four cultures—1.0×10^7 spleen cells initially cultured.

‡ Standard error.

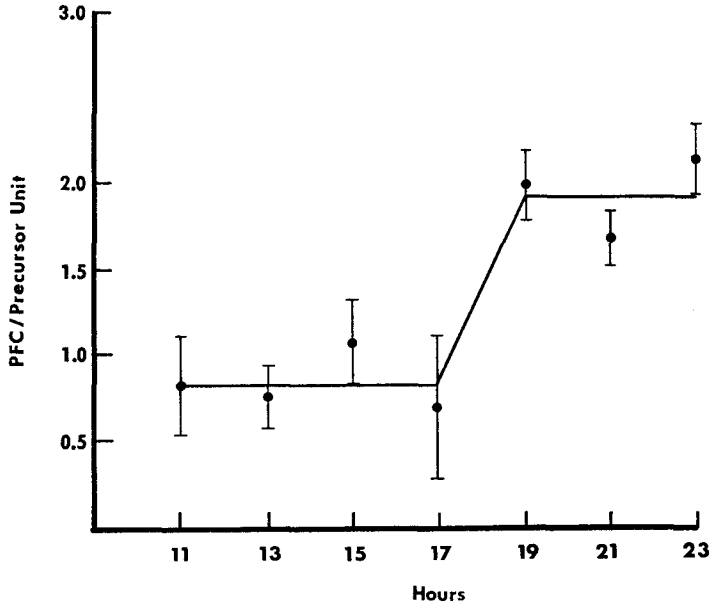

FIG. 2. Mean number of PFC/precursor unit measured at 2 hr intervals from 11–23 hr after initiation of culture in the presence of SRBC. Each point represents the mean of four cultures; the brackets represent the standard error of the mean.

depend on new DNA synthesis but that the increase of PFC after initial maturation of PPFC does depend on new DNA synthesis, suggesting that these cells (PFC) arise by division.

DISCUSSION

All conclusions drawn from these experiments pertain only to 19S hemolysin-producing cell clones. Before going into a discussion of how the present experiments may relate to previous reports concerning cell-cell interaction, I would like to first discuss questions which obviously arise concerning the technique itself. For example, do the PFC which develop all arise from those cells which

TABLE IV

Kinetics of Developing HPCC (18–32 hr)

Time	Precursor* units / culture		PFC* / culture		PFC / pre. unit		Approximate number doublings	
	Exp.		Exp.		Exp.		Exp.	
	1	2	1	2	1	2	1	2
hr								
18	—	12.5	—	24	—	1.92 ± .25‡	—	1
20	—	12.5	—	30	—	2.40 ± .25	—	1
22	11.5	11.5	27	42	2.35 ± .23‡	3.65 ± .41	1	2
24	15.0	14.0	56	41	3.73 ± .13	2.92 ± .09	2	1+
26	12.0	10.0	51	46	4.25 ± .62	4.60 ± .48	2	2
28	12.0	13.0	52	54	4.34 ± .18	4.15 ± .15	2	2
30	11.0	11.0	64	79	5.82 ± 1.1	7.17 ± .66	2+	3
32	12.0	11.0	108	84	9.00 ± 1.2	7.62 ± .66	3	3

* Mean of four cultures—1.1 × 10^7 spleen cells initially cultured experiment 1; 1.1 × 10^7 spleen cells initially cultured experiment 2.

‡ Standard error.

have started to make and release hemolysin within 4 hr after stimulation with antigen? Evidence which supports this view includes the fact that the initial increase in PFC which occurs between 17 and 19 hr is always about double the number of plaques seen on the culture plate. In addition, the fact that the number of large plaques on the culture dish remains essentially constant while the total PFC/culture increase exponentially also supports this view. However, by the second or third doubling of PFC, there is usually some displacement of hemolysin-producing cells as evidenced by the appearance of small plaques, often paired, immediately adjacent to the large precursor unit plaque. Those cells causing small plaques are probably derived from the larger parent plaque since small plaques do not appear randomly throughout the culture plate with time; they are always in close proximity to a large plaque. The movement of PFC from the parent plaque is possibly due to such vibrations which occur when

the warm room door is opened and closed, or when the heating unit shuts on or off. This phenomenon actually provides further evidence that the PFC do indeed arise in clones.

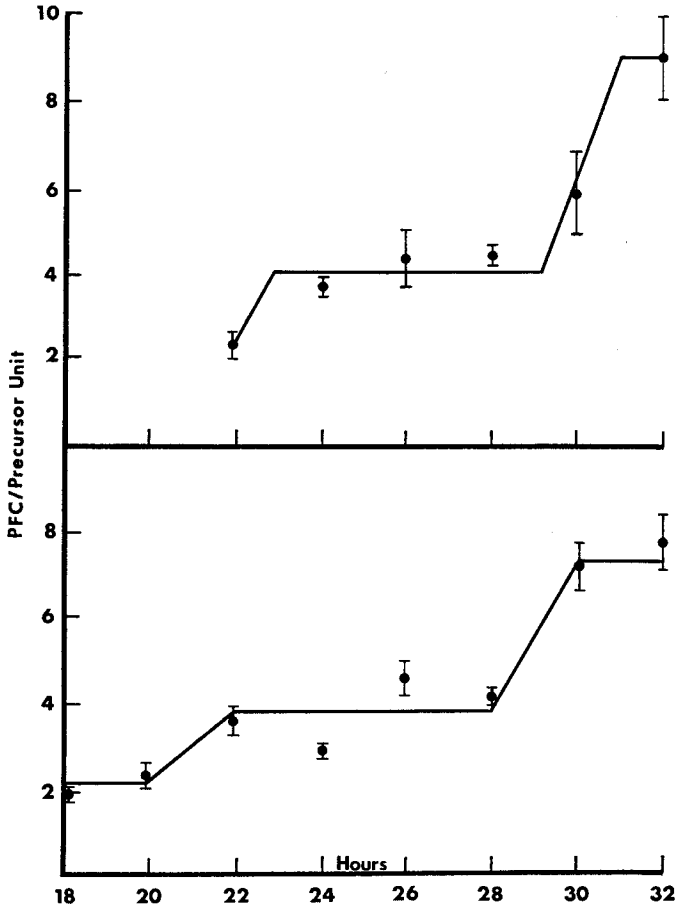

FIG. 3. Mean number of PFC/precursor unit measured at 2 hr intervals of two experiments. The first experiment (upper graph) records the interval from 22–32 hr after initiation of culture (1.1×10^7 cells/culture) in the presence of SRBC. The second experiment (lower graph) records the interval from 18–32 hr after initiation of culture (1.1×10^7 cells/culture) in the presence of SRBC. Each point represents the mean of four cultures; the brackets represent the standard error of the mean.

Another question which may be asked is: What is the relationship between so-called "background" cells and those cells which are responsible for the first plaques seen on the culture plate? This is a difficult question to answer and I

feel that the real significance of background cells has yet to be determined. However, in other experiments not reported here,[1] I have used this technique to follow the maturation of the hemolysin response in neonatal mice. These experiments show that mice younger than 2 days old have in their lymphoid tissues functional precursor units present which are capable, in the presence of

TABLE V

Effect of Puromycin Dihydrochloride on the Maturation of HPCC in Culture

Puromycin treatment	Precursor units / culture	PFC* / culture	Cell viability‡
μg/ml			%
None	21	224	80
1	15	92	75
6	7	3	70
12.5	3	2	70
25.0	1.5	0	25
50.0	0.5	0	15

* Mean of four cultures—1.5×10^7 spleen cells initially cultured; assayed at 48 hr.
‡ As determined by trypan blue exclusion at time of recovery.

TABLE VI

Effect of FUdR on the Maturation of HPCC in Culture

FUdR treatment	Precursor units* / culture	PFC* / culture	Cell viability‡
			%
None	25	170	75
10^{-3} M	28	20	65
10^{-4} M	33	23	70
10^{-5} M	26	19	75
10^{-6} M	25	90	70

* Mean of four cultures—1.5×10^7 spleen cells initially cultured; assayed at 48 hr.
‡ As determined by trypan blue exclusion at time of recovery.

antigen, of forming clones of hemolysin-producing cells in culture. I doubt seriously that such cells in mice of this age can be called "background" cells. In addition, new protein synthesis was required for the initial maturation of PPFC as evidenced by the puromycin data. However, if these early cells reported here are indeed "background" cells, and since they are capable of responding specifically to antigen to replicate and form large numbers of hemolysin-producing progeny, then I submit that "background" cells are PPFC.

[1] Saunders, G. C. 1969. Maturation of hemolysin producing cell clones. II. The appearance and localization of PPFC in lymphoid tissues of neonatal mice. In preparation.

The loss of exponential growth after 48 hr may be due to one or more of the following factors. (*a*) Excess antibody may shut off cell division. As these cultures are not rocked most of the antibody produced probably is diffused into the upper solid phase directly under the developing clone of hemolysin-producing cells. It would be reasonable to assume that as red cells in this area become saturated with antigen the subsequent presence of unbound antibody may by a feedback mechanism shut off further cell division. (*b*) Following similar reasoning, the loss, due to binding of the RBCs with antibody, of unaltered antigen adjacent to HPCC may also act to shut off all division. (*c*) These cultures are not fed and substances in the medium which promote cell division may become depleted. (*d*) An essential interaction between any cells which could cause continuation of replication may be prevented by the culture conditions. This system does provide means for determining the role of each of the above factors as they may pertain to shut off of exponential growth.

The observation that FUdR did not inhibit initial maturation of hemolysin-producing cells suggests that this early maturation does not require cell division. However, FUdR would not prevent cells in G2, which have already made their DNA, from dividing. However, the fact that each early precursor unit plaque contains only one assayable PFC does at least suggest that these cells have not divided. Shearer et al. (14) recently reported data which indicate that PPFC present in normal mouse spleens are in a relatively advanced differentiated state before they are initially stimulated with antigen. The present experiments support this view.

Further evidence to ascertain the validity or invalidity of maturation without division may be obtained with mitotic inhibitors. In any case, the synchronous doubling of hemolysin-producing cells observed in these experiments definitely implies that all PPFC that respond to antigenic stimulation must be in the same phase of the cell cycle when they are induced, most likely somewhere in G-1.

In contrast to the report of Mosier (15), cell clusters do form in this system in the absence of rocking. Microscopic observations of cells carefully pipetted from the cultures showed the presence of as many as one cluster per 300 cells counted as early as 4 hr after incubation began. The significance of the clusters in this system has not been determined. However, it does appear that if clusters are necessary for initial stimulation, maintenance of their integrity is not. As discussed earlier, progeny of PPFC do not necessarily remain as a single clump of cells.

The question of just what makes up a precursor unit cannot as yet be determined. One cell which must be present, however, is the immediate PPFC. Whether or not one, two, or more other cells are required in this system is not known at this time.

There is no question but that cell-cell interactions play a role in in vivo (16–

18) and in vitro (5, 8, 15, 19–22) antibody-producing systems. At least a two cell interaction is required in the transfer system first described by Claman et al. (16). Using this system (23–25), it has been postulated that antigen must somehow interact with a thymus derived cell, termed the antigen-reactive cell (ARC) before successful stimulation and maturation of the bone marrow derived immediate antibody-forming cell precursor (AFCP = PPFC) could take place. For this type of interaction to be true for the observation of the simultaneous stimulation of PPFC resulting in synchronous division reported here, certain conditions would have to exist. Either specific clusters would have to rapidly form in which both cell types (ARC and PPFC) were present, or one of the two cell types would have to be in large excess such that the odds that both cell types were close enough to each other to interact would be much greater. Macrophages could fit into this system in the following manner: interaction of the ARC with antigen may be followed by attachment of the activated reactive cell, or activated antigen, to a macrophage; the macrophage, being a mobile cell may then wander among cells until random contact with a PPFC takes place at which time the induction of the PPFC to maturate occurs. One must keep in mind, that in the present system, these steps must all take place within a 2 hr period.

An alternate explanation to that offered by Miller and coworkers to explain their data is the following: in the thymus-marrow transfer system all host PPFC are presumably inactivated by irradiation. Could it not be possible that the interaction between the marrow cell and thymus cell occurs at the level of the differentiation of the PPFC? For example the thymus cells may induce the differentiation of bone marrow stem cells into specific immunocompetent cells (22, 26, 27). This induction may or may not require the presence of antigen. Such a scheme would fit into the current data in that the simultaneous development of several PPFC into HPCC could be more easily explained. The possibility would exist in this scheme that macrophages are not absolutely essential for the induction of PPFC especially in the Marbrook system where a large amount of antigen is present. However, I feel that macrophages play an important, if passive, role in the in vivo situation. Conceivably they may serve to deliver a relatively small amount of antigen to those few cells available which are capable of specifically reacting to it, to induce the formation of clones of antibody-producing cells.

Which of the models discussed here is correct (if either) obviously cannot yet be determined by data currently available. Certain manipulations of the Marbrook system may shed additional light on the matter.

SUMMARY

Investigations of the induction period of an in vitro hemolysin response to sheep erythrocyte antigen revealed the following:

1. After antigen stimulation precursors of plaque-forming cells rapidly maturate to the point of hemolysin production.

2. Initial maturation probably occurs in the absence of cell division.

3. After initial maturation, a latent period of about 12 hr occurs before the first doubling of PFC.

4. At least the first three cell doublings are synchronous, with a generation time of 7–8 hr.

5. Synchronous cell division implies that all precursor cells are at the same point in the cell cycle when they are initially stimulated.

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