CELL POPULATIONS AND CELL PROLIFERATION IN THE IN VITRO RESPONSE OF NORMAL MOUSE SPLEEN TO HETEROLOGOUS ERYTHROCYTES*,‡

Analysis by the Hot Pulse Technique

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It has been shown in the accompanying paper (1) that spleen cell suspensions from normal mice can be immunized to heterologous erythrocytes in an in vitro system. When sheep erythrocytes are added at the initiation of culture, the number of antibody-forming cells (as determined by the hemolytic plaque assay) rises from an initial value of approximately 1 per 10^6 to 1,000 per 10^6 recovered cells 4 days later in a typical experiment. The kinetics of the response follow an approximately exponential form at least in the later time periods. The assay system measures 19S antibody–forming cells (2) and the response is "primary" in the sense that it follows the first experimental exposure to antigen.

Previous in vivo studies have indicated that the increase is largely the result of proliferation of precursor cells and they have further suggested that cell proliferation begins after an 18–24 hr lag period (for a review see reference 3). These studies, however, leave some room for doubt, as will be discussed below, and the problem has been reinvestigated in this in vitro system as part of a more general analysis of the cellular response to antigen. The questions asked here are: (*a*) at what times during the response are the precursors of the antibody-forming cells proliferating, (*b*) how many of the antibody-forming cells could have arisen by some transformation not involving cell division, and (*c*) are different cell populations involved in the simultaneous response to two non–cross-reacting groups of antigens? The procedure that has been used, and which we have termed the "hot pulse" technique, is to expose the cell population to tritiated thymidine of very high specific activity for defined periods of time. DNA-synthesizing (proliferating) cells concentrate a sufficient quantity of radioactivity to acquire an effective dose of irradiation in a brief period of time. Non-

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dividing cells are uninjured, since they do not concentrate thymidine and are exposed to a much lower dose of irradiation.

The effect of such "hot pulses" administered at various times on the subsequent appearance of antibody forming cells was determined. The results show that (*a*) the onset of DNA synthesis does not start for 24 to 32 hr after the addition of antigen, (*b*) essentially all the antibody-forming cells arise by cell division, and (*c*) that different populations of cells are involved in the concurrent responses to two non–cross-reacting antigens.

Materials and Methods

The conditions for the preparation, culture, and immunization of mouse spleen cells, and the hemolytic plaque assay for antibody forming cells, are described in the accompanying paper (1). The hot pulse technique is described below. Tritiated thymidine at 13 or 15 c/mM and cold thymidine were obtained from Schwarz Bio Research Inc., Orangeburg, N. Y.

Hot Pulse Technique.—The hot pulse technique is a procedure designed to kill dividing cells and have no effect on nondividing cells. To achieve this end, the following requirements must be met. (*a*) The radioactivity incorporated into the DNA of a dividing cell must be sufficient to bring about a lethal irradiation of the cell in a brief period of time. (*b*) The nondividing cells should be exposed to a small enough dose of radioactivity so that they suffer no demonstrable harm. Some preliminary calculations served to indicate the possibility of achieving such an effect by the addition of tritiated thymidine of high specific activity to the medium.[1] In practice, however, an effective dose of tritiated thymidine was found empirically and was shown experimentally to provide the required selective effect. The effect that was measured was the subsequent nonappearance of antibody-forming cells and the proof that this was a consequence of thymidine incorporation rested on the demonstration that the effect was blocked by the simultaneous addition of excess cold thymidine. Such cultures contained the same total radioactivity, but the specific activity of the thymidine, and hence the incorporation of radioactivity, was reduced 300-fold (see below).

10 μc of tritiated thymidine in 0.16 μg (15 c/mM) per 1 ml culture was found satisfactory for the hot pulse and an aditional 100 μg of cold thymidine was added for the blocked pulse. To terminate the pulse the cultures were harvested and spun for 10 min at 1000 rpm. The supernatants were discarded and the cells resuspended in 5/6ths of the original volume (to compensate for cell loss) of "conditioned medium" containing 50 μg/ml cold thymidine. The

[1] The cultures contain 1.5×10^7 cells in 1 ml. The tritiated thymidine becomes distributed between two compartments: (*a*) the total volume of the medium, and (*b*) the nuclei of the dividing cells. The volume of the first compartment is approximately 1 ml and that of the second is 10^{-4} ml (assuming that 1% of the cells divide and the volume of a cell nucleus is 500 μ^3). The "thymidine content" of a single cell is of the order of 3×10^{-6} μg or 0.45 μg for 1.5×10^5 cells. Thus, if exogenous thymidine were the sole source of DNA thymidine, most of 0.5 μg of added thymidine would be concentrated into 1/10,000 part of the total volume of the culture. The β particles of the disintegrating tritium are sufficiently soft so that the bulk of the energy of the incorporated activity is dissipated in the volume of the nucleus. It is easy te see that if the above assumptions were true, the procedure would afford an enormously selectivo method of irradiating dividing cells.

In reality, exogenous thymidine does not represent the sole source of DNA thymidine and it is not possible to arrive at any valid figure for the actual or relative doses of irradiation received by dividing and nondividing cells.

cell suspensions were reincubated in 1 ml cultures as before. The "conditioned medium" was prepared by harvesting the supernatants from 24 hr cultures of mouse spleen cells. The negative control and positive control cultures were treated in the same way. It was shown that the addition of 100 μg/ml cold thymidine had no effect on the development of plaque-forming cells (PFCs) during 4 days' culture.

The design of a typical experiment is summarized in Table I.

The Effect of 24 Hr Hot Pulses.—Stimulated spleen cell cultures were hot pulsed in the in vitro response at 0–24, 24–48, 48–72, or 72–96 hr after the addition of antigen. For the 0–24 hr pulses, the cultures were preincubated for 24 hr before the addition of antigen and tritiated thymidine to minimize possible effects of the transfer of the cells to in vitro conditions. In all other experiments, the antigen was added at time 0. The number of PFCs was assayed on day 4 and in some experiments on day 5 also. The results are presented in Tables II, III, IV and V.

TABLE I

Protocol for 24–48 Hr Hot Pulse Experiment

Mouse spleen cell suspension prepared

Sheep RBC

10 μc TdR* (15 c/mM)

±100 μg cold TdR

Pulse period

Harvest cells, resuspend in conditioned medium containing 100 μg/ml cold TdR, reincubate

Assay for PFCs

Day	0	1	2	3	4

Culture	Additions to medium		
	S-RBC	Hot TdR at 24 hr	Cold TdR at 24 hr
Negative control	−	−	−
Positive control	+	−	−
Hot pulse	+	+	−
Blocked pulse	+	+	+

* TdR, tritiated thymidine.

RESULTS

The presence of high specific activity thymidine during the first 24 hr period (Table II) had little or no effect on the subsequent development of plaque-forming cells.

It can be seen that hot pulsing in the 24–48 hr (Table III) or the 48–72 hr (Table IV) period reduced the number of plaque-forming cells to less than 3% of the stimulated control. Hot pulses in the period 72–96 hr (Table V) were less inhibitory when assayed at day 4. The degree of inhibition had increased, however, when the assay was made at day 5.

Duration of the Pulse.—Experiments were conducted to determine the

relationship between the duration of the pulse and the degree of inhibition of the development of PFCs. The time chosen was the period 24–48 hr after antigen addition and all pulses were terminated at 48 hr. It can be seen from Table VI that 6 hr was the minimum pulse time for complete inhibition under these conditions. (It is possible that different results for the minimum pulse period might be obtained for pulses delivered in another phase of the response or by using different amounts or specific activities of tritiated thymidine.)

Effect of 0–24 Hr Hot Pulse on the Response of 3 day In Vivo–Primed Cells

TABLE II

Effect of Hot Pulse Present in the Period 0–24 Hr after the Addition of Antigen on the Development of Plaque-Forming Cells

	Experiment No. 1316	Experiment No. 1330
Negative control	9	61
Positive control	530	600
Hot pulse	685	500
Blocked pulse	650	550

In these experiments, the cultures were preincubated for 24 hr before the addition of antigen. The number of PFCs per 10^6 recovered cells were assayed 4 days after the addition of antigen. Comparable cell recoveries were obtained in all cultures.

TABLE III

Effect of Hot Pulse Present in the Period 24–48 Hr after the Addition of Antigen

	Experiment No. 1305
Negative control	152
Positive control	1880
Hot pulse	42
Blocked pulse	1160

The number of PFCs per 10^6 recovered cells were assayed on day 4. Comparable cell recoveries were obtained in all cultures.

(*3 Day PR*).—Mice were injected with antigen 3 days before spleen cell preparation. Such cultures can be further stimulated by the addition of more erythrocytes at time 0 in culture, as described in the accompanying paper (1). The results in Table VII show that a 0–24 hr pulse given to such cultures greatly suppresses the response, in contrast to the finding with "unprimed" cells, noted above. This experiment is included only to demonstrate that there is no special feature of the 0–24 hr culture conditions that neutralizes the effect of the hot pulse.

The Effect of Hot Pulse on Responses to Two Non-Cross-Reacting Red Cells.—Burro erythrocytes were added to spleen cell suspensions from normal mice

at time 0. Sheep erythrocytes and a hot pulse were added at 24 hr. Thus, in the same culture, the response to burro cells was pulsed at 24–48 hr after antigen addition, while the response to sheep cells was pulsed at 0–24 hr. The results in Table VIII demonstrate that the inhibition of the burro response obtained with a 24–48 hr pulse and the lack of inhibition of the sheep response with a 0–24 hr pulse occur completely independently in the same cell suspensions. The same result was obtained when the first antigen (burro erythrocytes) was reintroduced at the same time as the hot pulse.

TABLE IV

Effect of Hot Pulse Present in the Period 48–72 Hr after the Addition of Antigen

	Experiment No. 1291
Negative control	57
Positive control	730
Hot pulse	8
Blocked pulse	760

The number of PFCs per 10^6 recovered cells were assayed on day 4. Comparable cell recoveries were obtained in all cell cultures.

TABLE V

Effect of Hot Pulse Present in the Period 72–96 Hr after the Addition of Antigen

	Experiment No.					
	1293	1299	1366		1374	
	Day 4	Day 4	Day 4	Day 5	Day 4	Day 5
Negative control	55	34	—	—	—	—
Positive control	550	404	1443	4300	208	1160
Hot pulse	430	208	606	180	144	99
Blocked pulse	500	478	—	—	172	720

The number of PFCs per 10^6 recovered cells were assayed on day 4 and day 5. Comparable cell recoveries were obtained in all cell cultures on a given day.

Duration of the Lag Period during Which No Inhibition Can Be Obtained with the Hot Pulse.—The duration of the lag period before the onset of DNA synthesis was more sharply defined by the use of 8 hr pulses. The experiments showed that no inhibition was obtained in any period up to 24 hr. The 32–40 hr pulse was the earliest pulse that was completely inhibitory (Table IX).

DISCUSSION

The cells that synthesize 19S antibody 4 days after the first experimental injection of antigen have been identified in different experimental models by

TABLE VI

Relationship between the Duration of the Hot Pulse and the Number of PFCs Determined at Day 4 or 5

	Experiment No.		
	412	417	1325
	Day 5	Day 5	Day 4
Negative control	—	—	32
Positive control	524	1836	420
24 hr pulse	7	13	20
24 hr pulse, blocked	—	—	550
18 hr pulse	—	—	22
18 hr pulse, blocked	—	—	460
12 hr pulse	—	—	15
12 hr pulse, blocked	—	—	270
8 hr pulse	5	15	—
6 hr pulse	29	3	39
6 hr pulse, blocked	—	—	310
4 hr pulse	93	218	—
2 hr pulse	186	418	—

All pulses end at 48 hours. The number of PFCs per 10^6 recovered cells were assayed on day 4 or day 5. Comparable cell recoveries were obtained in all cultures within each experiment.

TABLE VII

Effect of 0–24 Hr Hot Pulse on the Response of 3 Day PR Cells to Sheep Erythrocytes

	ExperimentNo.	
	392	394
Negative control	1,944	1,264
Positive control	17,400	17,625
Hot pulse	350	192
Blocked pulse	14,718	28,909

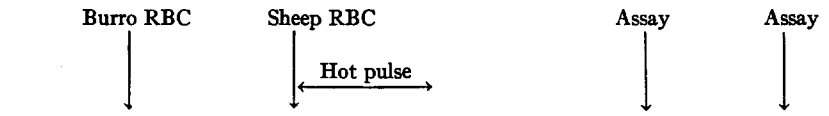
Mice were injected i.v. with 0.2 ml of a packed cell suspension of sheep erythrocytes 3 days before spleen cell suspension. The number of PFCs per 10^6 recovered cells was determined at day 3 (of culture). Comparable cell recoveries were obtained in all cell cultures.

a variety of techniques. Harris and his colleagues (4) carried out an electron microscopic analysis of the cells found at the centers of hemolytic plaques in the Jerne assay of mouse spleen cells responding to sheep erythrocytes. The hemolytic plaque-forming cells exhibited a wide range of morphological characteristics within the two general categories of lymphocyte and plasma cell

TABLE VIII

The Effect of a Hot Pulse on the Simultaneous Response to Two Non–Cross-Reacting Antigens, the Second Antigen Being Added 24 Hr after the First

Experimental protocol



Result

	Immunizing erythrocyte							
	Sheep only		Burro only		Sheep and burro			
Erythrocyte for assay	Sheep		Burro		Sheep		Burro	
Day	4	5	4	5	4	5	4	5
Negative control	32	4	0	2	32	4	0	2
Positive control	93	700	78	210	103	445	88	187
Hot pulse	90	750	0	6	73	460	0	8
Blocked pulse	140	330	88	77	150	960	97	330

TABLE IX

Duration of Lag Period Before the Onset of DNA Synthesis

	Experiment No.			
	1360	1361	1369	1370
Negative control	11	44	—	—
Positive control	245	360	323	244
8–16 hr pulse	230	200	—	—
16–24 hr pulse	390	120	242	200
24–32 hr pulse	75	218	65	37
32–40 hr pulse	17	44	26	22

The number of PFCs per 10^6 recovered cells were assayed on day 4. Comparable cell recoveries were obtained in all cultures.

classes. Since antibody-forming cells are not present in anything like comparable numbers prior to stimulation, they must arise either (*a*) by intense proliferation of the very small numbers of plaque-forming cells that can be shown to be present initially, (*b*) by proliferation and differentiation of some other unidentified precursor cell population, (*c*) by differentiation (without

division) of a much larger cell population, or (*d*) by some combination of the three preceding possibilities. Previous studies have narrowed the range of possibilities and most workers would exclude possibility number three, but protagonists can be found for each of the other hypotheses.

A wealth of evidence has been presented by a variety of techniques for proliferation in the secondary response (5–20) and also concerning the primary response (5, 9–11, 21–27, 29). This literature has been recently reviewed by the authors (3) and will not be further discussed other than to say that it indicates conclusively that proliferation plays some part in the development of the antibody-forming cell population.

The evidence concerning the extent of proliferation and the question as to whether all the cells are derived by proliferation is less clear cut. In the studies of Schooley (10) and Nossal and Mäkelä (11), the initial thymidine-labeled cell population increased up to 1000-fold. Similar conclusions as to the extent of proliferation have been reached from the rise in the number of plaque-forming cells following antigenic stimulation (2, 27). Doubling times in the number of antibody cells less than 6 hr have been found, however (28), and this has led some to conclude that this data cannot be interpreted solely in terms of cell proliferation. It should be pointed out that these data do not demand a *generation* time of 6 hr or less if the antibody-forming cells are derived from the proliferation of a somewhat larger number of non–antibody-forming precursor cells. Doubling times of plaque formers as short as 4 hr are not incompatible with the conclusion that all the antibody-forming cells arise by cell division.

Koros et al. (29) have shown that 55% of the hemolytic plaque-forming cells were labeled after 30 min incubation with labeled thymidine immediately prior to assay. They calculated from this observation and the doubling time of the number of plaque-forming cells a generation time of 7 hr and an S period of 4 hr. Others have concluded from the inability of a single pulse of thymidine to label all the subsequently observed antibody-forming cells, and for other reasons, that the unlabeled cells are derived by differentiation without proliferation (30).

It was the purpose of this present study to define the role of proliferation in the response in an in vitro system, and only incidental and inconclusive evidence is presented on the role of differentiation.

The hot pulse technique provides for a selective irradiation of any cell that is synthesizing DNA during the defined period of time of the pulse. The proof of this selectivity is the reversal of the effect by dilution of the thymidine by the simultaneous addition of excess cold thymidine. Cold thymidine can itself have an inhibitory effect on DNA synthesis, and it was somewhat surprising to find that in this system 100 μg/ml cold thymidine had no inhibitory effect on the appearance of plaque formers. The inhibitory effect of the hot pulse is

clearly due to some consequence of the radioactivity of the incorporated thymidine. It is not known, however, whether the cell is prevented from further division or dies at the next replication of DNA; and it is not known whether or when its somatic functions are affected. A more primitive version of the technique was used in an earlier study (7).

The use of this technique indicates that the production of virtually all the antibody-forming cells depends on continuous cell proliferation starting 24–32 hr after antigen addition. Three explanations of this effect appear possible. (*a*) The antibody-forming cells are derived directly from proliferation of precursor cells that are killed by the hot pulse. (*b*) The hot pulse kills a small number of proliferating cells which releases a nonspecific, inhibitory factor which in some way prevents the development of the response. (*c*) The hot pulse kills a small number of proliferating cells which hand on a specific message [e.g., as might occur in the "antibody virus" hypothesis put forward by Smithies (31)] to other cells which then transform with or without further proliferation into antibody-forming cells. The second possibility is excluded by the experiment with the two non–cross-reacting antigens. The hot pulse that prevents the response to the antigen added 24 hr earlier has no effect on the response to the second antigen added with the hot pulse. The third possibility is not excluded by any of the experiments presented. It involves, however, a complex scheme of events for which there is no evidence. It would, therefore, seem more likely that the antibody-forming cells are derived directly from the proliferation of specific precursor cells and that virtually no cells arise by transformation without proliferation.

The minimum pulse period for complete inhibition was found to be 6 hr when pulses ended at 48 hr. It is entirely possible that different values would have been found at other stages of the response. This time defines the minimum time required for all the dividing cells to take up a lethal dose of radioactivity. It does not define the mean generation time of the cells.

Previous studies have indicated a very considerable restriction in the number and class of antibodies that can be synthesized by a single cell at any given moment. O'Brien and Coons (8) showed that separate cell populations were involved in a simultaneous secondary response to two antigens.

The present data are in accord with and extend the above observations, showing that the two responses (each presumably to a group of antigens) involve entirely separate precursor cell populations present during the early stage of the response. Furthermore, the result of the experiment implies that they were distinct and present before the first experimental exposure to the antigen. The observation may thus be taken to support the hypothesis that committed cells exist prior to contact with antigen. The argument is not watertight, however, since (*a*) it is not clear that the animals have not been previously exposed to cross-reacting antigens in the environment, and (*b*)

more complex hypotheses not involving preexistent cell populations can be devised.

The lag period before the onset of DNA synthesis (in this response to an erythrocyte antigen) has been provisionally defined as 24–32 hr. This finding must be accepted with some reservation, since low responses were observed in these particular experiments. It is possible that the duration of the lag period is dependent on culture conditions. The experiments with the primed response (Table VII) and the double antigen (Table VIII) indicate that this lag period is not an artefact of the early period of the in vitro culture, but has some biological significance. Similar lag periods have been established in in vivo experiments by Sterzl et al. (27) and by various studies with inhibitors of DNA synthesis (24, 25). There is no indication in the present experiments as to what events occur during the lag period.

SUMMARY

The role of proliferation in the development of 19S antibody–forming cells in the primary response has been investigated in an in vitro system.

Spleen cell suspensions from normal, unimmunized mice were cultured in vitro in the presence of mammalian erythrocytes and the number of 19S hemolytic plaque-forming cells that arose 4 days later was measured. The hot pulse technique for the selective irradiation of those cells which synthesize DNA during a defined period of time has been described.

The effect of such hot pulses administered at various times after the addition of antigen on the subsequent appearance of antibody-forming cells was determined.

The results established that: (*a*) the onset of DNA synthesis does not start for approximately 24–32 hr after the addition of antigen, (*b*) essentially all the antibody-forming cells arise by cell division, and (*c*) different cell populations are involved in the response to two non–cross-reacting antigens.

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