STUDIES ON ANTIBODY PRODUCTION

VII. The Effect of 5-Bromodeoxyuridine on the in Vitro Anamnestic Antibody Response*

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5-Bromodeoxyuridine (BUDR) is a thymidine analogue with a bromine atom substituted for the 5-methyl group of thymidine. It is acceptable as a substrate to the phosphorylating and polymerizing enzymes that synthesize deoxyribonucleic acid (DNA) (1–6), and in the resulting DNA molecule it may replace in various organisms more than half of the thymidine normally incorporated (3). This abnormal DNA is associated with an increased mutation rate in viruses and bacteria (7, 8). The multiplication rate of cultured mammalian cells approaches zero as the percentage of BUDR incorporated into the DNA is increased (3, 6), probably because the genetic information has been so falsified (9–11) that a viable daughter cell is no longer probable.

DNA synthesis appears to occur in a mammalian cell only when the cell is about to divide; there is no appreciable incorporation of thymidine, or presumably of its analogues, into the DNA of a resting cell (12–15). This is the basis for the widely accepted principle that the incorporation of labeled thymidine into the nucleus of a cell means cell division impending or accomplished after exposure to the labeled base.

By the same reasoning, sufficient BUDR added to a population of mammalian cells for a limited period might eliminate selectively only the progeny of those cells that were in the process of dividing during that period. Such selectivity would provide a method for analyzing, in a mixed cell population, the time and circumstances of cell division, particularly the division of obscure progenitive cells, the progeny of which attain some measurable activity. This was the rationale for the following experiments in which BUDR was added at various stages in its development to an anamnestic antibody response system *in vitro*.

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Materials and Methods

Assay System.—Roller tube cultures were made from fragments of popliteal lymph nodes excised from rabbits which had been immunized once approximately 3 months earlier with both bovine serum albumin (BSA) and diphtheria toxoid (D). Before or after implantation most of the fragments were "stimulated" by incubation with antigen *in vitro*. Antibody in the culture medium was titrated by the passive hemagglutination method of Boyden. The materials and methods of this *in vitro* system have been described in detail (16).

BUDR Exposure.—5-bromodeoxyuridine (California Corporation for Biochemical Research, Los Angeles) was dissolved in phosphate-buffered normal saline (pH 7.2) at a concentration of 5 mg/ml and passed through a Millipore filter for sterilization. Unless otherwise specified, 0.02 ml was added to 1 ml of culture medium in each tube exposed to BUDR, for

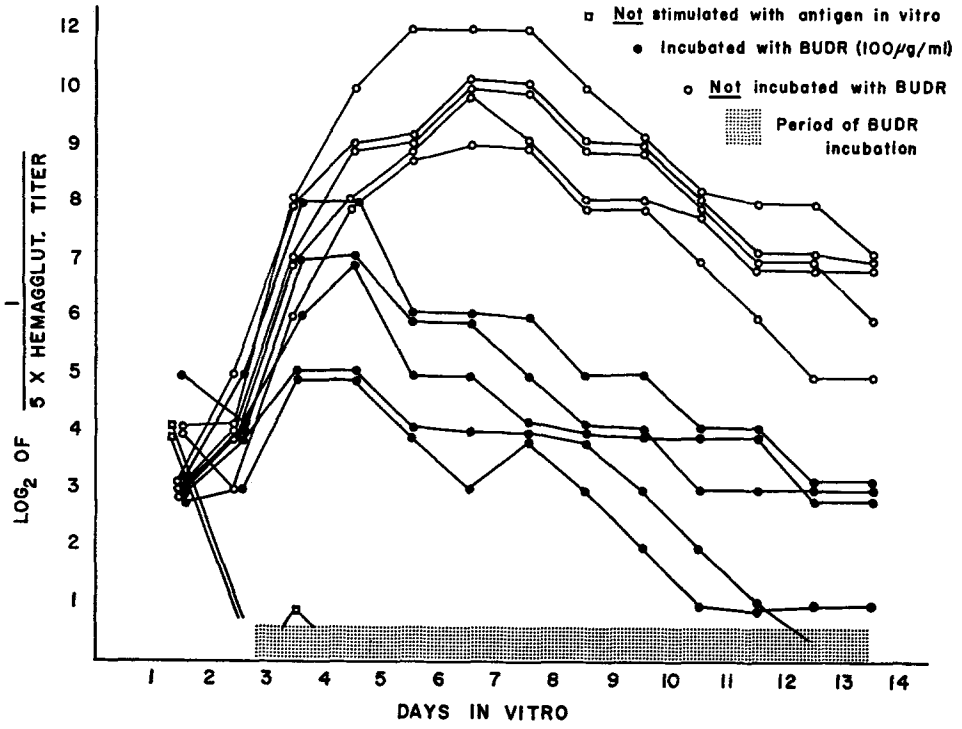
TABLE I

The Effect of BUDR on Antibody Production in Vitro

Day *in vitro*	Anti-diphtheria			Anti-BSA		
	0 to 4	4 to 7	7 to 10	0 to 4	4 to 7	7 to 10
Not stimulated with antigen	20	10	0	160	40	40
	0	0	0	80	20	0
Stimulated but not exposed to BUDR	40	640	160	1,200	10,000	2,500
	40	1,200	320	320	10,000	2,500
	160	640	320	640	10,000	2,500
	160	1,200	640	640	10,000	5,000
	20	640	640	640	5,000	2,500
BUDR exposure begun 1 day after stimulation	0	0	0	20	0	0
	0	0	0	20	0	0
	0	0	0	80	10	0
	0	0	0	40	0	0
	0	0	0	40	0	0
BUDR exposure begun 2.5 days after stimulation	80	80	—	320	320	—
	40	80	80	160	160	160
	40	40	10	320	320	160
	40	40	20	160	80	80
	20	20	10	160	80	80
BUDR exposure begun 4 days after stimulation	40	640	—	160	2,500	—
	20	320	160	320	2,500	2,500
	160	640	640	640	5,000	2,500
	40	320	320	640	5,000	2,500
	80	640	320	320	2,500	2,500

Antibody titers to each of 2 antigens in culture medium changed at 3 or 4 day intervals in 22 tubes, 20 of which were stimulated *in vitro* with both antigens at the time of implantation. BUDR (100 μg/ml) was present in 15 tubes from the times indicated until the end of the experiment. Dashes (—) represent 2 tubes broken before final titration.

a final concentration of approximately 100 μg/ml. At the end of the period of exposure to BUDR, all of the culture medium was aspirated from each of the tubes with a micropipette. The tubes and fragments were then washed twice by the addition of 2.5 ml of Hanks' balanced salt solution (pH 7.4) to each of the tubes, rotating in the roller drum for 5 minutes, and then pouring off the solution.



TEXT-FIG. 1. Anti-BSA titers of culture medium changed daily in each of 12 tubes. Of the 10 tubes stimulated with antigen at the time of implantation, 5 had BUDR at 100 μg/ml in their medium from the 70th hour *in vitro* to the end of the experiment (Experiment 25).

RESULTS

Time of BUDR Exposure.—Table I shows the typical results of prolonged exposure of this *in vitro* system to BUDR. Tubes in which BUDR exposure was begun 1 day after the *in vitro* stimulation and continued to the end of the experiment have essentially no detectable antibody production to either of the two antigens used. Tubes in which BUDR exposure was begun 2.5 days after the antigen stimulation and continued to the end of the experiment have a substantially reduced antibody production. Tubes in which exposure to BUDR is begun 4 days after antigen stimulation and maintained to the end of the experiment have little or no significant impairment of antibody production.

Fig. 1 shows an experiment in which antibody production was measured daily instead of every 3rd day. 70 hours after the *in vitro* stimulation with antigen, 5 tubes were exposed to BUDR, an exposure which was continued throughout the experiment. As in Table I the tubes incubated with BUDR

TABLE II

The Effect of BUDR During Restricted Intervals

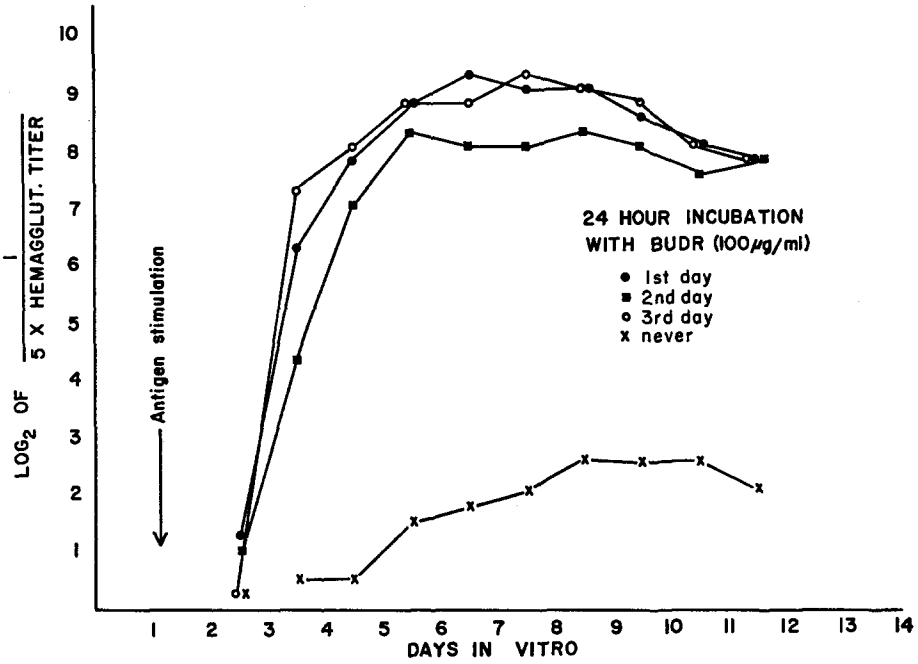
Day *in vitro*.....	Anti-diphtheria				Anti-BSA			
	0 to 3	3 to 7	7 to 10	10 to 14	0 to 3	3 to 7	7 to 10	10 to 14
Unstimulated	0	0	0	0	40	640	640	320
controls	0	0	0	0	20	10	160	640
Stimulated but	0	640	640	80	80	10,000	10,000	1,200
not exposed to	0	640	640	80	80	20,000	20,000	5,000
BUDR	0	160	160	10	40	10,000	10,000	1,200
	0	320	160	160	40	10,000	10,000	5,000
	0	160	320	40	80	10,000	20,000	640
Exposed to	0	160	160	20	10	5,000	5,000	640
BUDR for 1st	0	320	640	80	20	5,000	10,000	1,200
day after stim-	0	160	80	20	20	5,000	5,000	1,200
ulation	0	320	320	40	40	2,500	5,000	1,200
Exposed to	0	0	10	0	10	160	320	80
BUDR for 2nd	0	0	40	20	0	80	320	80
day after stim-	0	0	20	0	0	80	160	40
ulation	0	0	40	0	0	80	640	160
Exposed to		0	10	10		40	40	20
BUDR for 3rd		0	0	0		80	80	20
day after stim-		0	20	10		80	40	20
ulation		0	0	0		80	40	20

Antibody to each of two antigens titered in culture medium changed at 3 or 4 day intervals in 19 tubes. Of the 17 tubes stimulated with antigen at the time of implantation, 12 were exposed to BUDR on either the 1st, 2nd, or 3rd day after stimulation.

during this period had a substantially reduced rate of antibody production. However, this antibody production did persist, tapering off gradually on a slope parallel to that of the normal response. As reported previously (17), sections from the "normal response tubes in this experiment showed numerous cells containing antibody when examined by immunofluorescence. Sections from the tubes exposed to BUDR showed a few isolated cells containing antibody.

Table II presents the results of an experiment in which BUDR was added

in single pulses of 24 hours duration at various times. Exposure to BUDR during the first 24 hours after stimulation had little effect on subsequent antibody formation. However, when it was present during the second 24 hours after stimulation, it reduced subsequent antibody production substantially and caused a still greater reduction when it was present during the third 24 hour period.



TEXT-FIG. 2. An experiment in which antigen stimulation occurred 1 day after implantation. 4 tubes were exposed to BUDR on each of the first 3 days after implantation, and 4 tubes were not exposed. Presented here for each category is the average of $\log_2$ anti-BSA titers in culture medium changed daily (from Experiment 30).

To eliminate the possibility that these results depended on the time of implantation of the fragments rather than on the time of antigen stimulation, another experiment was performed (Fig. 2) in which antigen stimulation was delayed until 24 hours after implantation. Again BUDR exposure during the 1st day after antigen stimulation had little effect, while BUDR exposure during the 2nd day after antigen stimulation substantially inhibited subsequent antibody production. This experimental design also permitted BUDR exposure for the 24 hour period following implantation but prior to antigen stimulation. This had no appreciable effect on the ensuing antibody production. In later experiments (Table VI) the period between implantation and antigen stimula-

tion was extended to 2 days. BUDR exposure throughout this period appeared to have no significant effect on subsequent antibody production.

Since the anamnestic antibody response *in vitro* seemed most susceptible to BUDR suppression during the 2nd and 3rd days after antigen stimulation,

TABLE III

Differing Lengths of Exposure to BUDR

	Anti-diphtheria	Anti-BSA
Unstimulated contsols	0	160
	0	80
Not exposed to BUDR	640	2500
	640	2500
	320	640
	160	640
Exposed to BUDR for 2 hours (hours 44 to 46)	320	640
	640	1200
	640	640
	640	640
Exposed to BUDR for 6 hours (hours 44 to 50)	80	320
	320	640
	320	640
	640	640
Exposed to BUDR for 12 hours (hours 44 to 56)	0	80
	80	80
	320	320
	80	160
Exposed to BUDR for 24 hours (hours 44 to 68)	0	10
	0	40
	0	20
	0	80

Anti-diphtheria toxoid and anti-BSA titers of culture medium incubated in 22 tubes from the 4th to the 7th day. Of 20 tubes stimulated with antigen at the time of implantation, 16 were exposed to BUDR at 100 μg/ml for varied lengths of time, all beginning 44 hours after implantation.

an attempt was made to determine the minimum effective duration of BUDR exposure during this period (Table III). 12 hours' exposure appeared to cause a reduction in subsequent peak antibody formation but the reduction was more striking after 24 hours of BUDR exposure.

In most experiments, the final BUDR concentration was usually 100 μg/ml.

Table IV indicates the minimum concentration of inhibitor which is effective when present from the 2nd through the 4th day after antigen stimulation. 6 μg/ml during this 3 day period was sufficient to cause a substantial reduction in subsequent antibody production.

TABLE IV

Dose-Response Effect of BUDR

	Anti-diphtheria	Anti-BSA
Unstimulated controls	0	20
	0	10
Not exposed to BUDR	640	5000
	320	1200
	640	1200
	320	1200
Exposed to BUDR, 1.5 μg/ml	160	640
	80	640
	320	1200
	320	1200
Exposed to BUDR, 6 μg/ml	20	40
	0	0
	0	20
	0	20
Exposed to BUDR, 25 μg/ml	0	20
	0	0
	0	10
	0	20
Exposed to BUDR, 100 μg/ml	0	0
	0	0
	0	10
	0	20

Titers of antibody to 2 antigens in culture medium incubated in 22 tubes from the 4th to the 7th day *in vitro*. Of the 20 tubes stimulated with the antigens at the time of implantation 16 were exposed to varied concentrations of BUDR during the 2nd, 3rd, and 4th days *in vitro* (Experiment 38).

Table V shows the antibody titers to each of two antigens on the day of peak antibody production, the 8th day after implantation, in a different type of experiment. Except for the unstimulated control tubes, all of the tubes were stimulated with both antigens, BSA and diphtheria toxoid, at a concentration of 10 μg/ml for a 4 hour period 2.5 days after implantation. Some of the tubes

TABLE V

The Effect of a Pulse of BUDR

Incorporated into culture medium			Antibody production on 8th day	
BSA for first 2.5 days	BUDR for first 2.5 days	Diphtheria toxoid and BSA for 4 hrs. at end of first 2.5 days*	Anti-diphtheria toxiod	Anti-BSA
No	No	No	0	640
			0	360
			0	360
			10	640
No	Yes	No	0	10
			0	20
			0	20
			0	20
No	No	Yes	320	1200
			160	2500
			320	2500
			320	2500
			160	1200
			320	1200
No	Yes	Yes	80	1200
			160	1200
			320	2500
			80	1200
			80	1200
			80	1200
			80	2500
			80	1200
Yes	Yes	Yes	320	40
			320	40
			160	80
			160	80
			40	20
			320	80
			160	80
			320	80

Titers of antibodies to 2 antigens in culture medium removed from the tubes of Experiment 39 at the end of the 8th day *in vitro*. The special composition of the medium for the first 2.5 days *in vitro* is outlined. After the first 2.5 days the usual medium was used and changed daily.

* For this *in vitro* system, the later the antigen stimulation, the smaller the antibody response. Antigen during the 1st day undoubtedly would have stimulated these fragments to greater antibody production than did the stimulation at 2.5 days required by this experiment. This probably would restore the ratio of antibody production in stimulated and unstimulated tubes to the higher values usually seen (17) and seen here when both were exposed to BUDR for the first 2.5 days. Note that BUDR suppresses the unstimulated response.

Previous experiments (16) have excluded the possibility that prolonged exposure to the antigen might in itself be inhibitory.

were also exposed to BUDR for the 2.5 day period prior to antigen stimulation and these tubes had no significant impairment of subsequent antibody production. However, tubes that were exposed to BUDR plus one of the antigens (BSA, 10 μg/ml) during this 2.5-day period showed a severe reduction in the subsequent production of antibody to that antigen, but an unimpaired antibody response to the other antigen.

DISCUSSION

These experiments define a time period in the course of the anamnestic antibody response *in vitro* during which exposure to a relatively low concentration of 5-bromodeoxyuridine (BUDR) severely damages the antibody response, but before or after which exposure to a much higher BUDR concentration has little effect. This period includes the 2nd, 3rd, and probably 4th days after antigen stimulation.

Certain cells in the culture actively produce specific antibody after this BUDR-sensitive period. Prior to the BUDR sensitive-period, some cells in the culture must possess and retain the potential for developing into or giving rise to cells producing specific antibody. The inability of BUDR even in high concentrations, to interfere measurably with either of these complex capabilities except during the susceptible period supports the theoretical expectation outlined in the introduction that BUDR would be toxic for cells only during or just prior to mitosis. Cell division with its associated DNA replication appears to offer a BUDR molecule its only opportunity for incorporation into the DNA of a cell. Since this analogue seems to have no other appreciable effect on cellular function (4), such incorporation would seem to provide its only chance to damage the function or progeny of a cell. This suggests that during and only during, the BUDR-sensitive period is an appreciable rate of cell division occurring in the cell line responsible for specific antibody production.

Other observations support this view. The susceptible period occurs during and just antecedent to a time when cells containing antibody are becoming numerous and antibody production is accelerating most rapidly. Conclusive evidence has accumulated (18–21) that rapid cell multiplication occurs during this period of the *in vivo* anamnestic response, which the *in vitro* system closely resembles (17). Moreover, in this susceptible period the minimal duration of BUDR exposure required for an appreciable reduction of antibody production, 12 to 24 hours, is in the same order of magnitude as estimated for one or two generation times for such cells (18, 20). In a population of cells dividing continuously and asynchronously, a generation time is the shortest period that would encompass one round of DNA replication in all the cells and thus, theoretically, the shortest period that could provide unifilar incorporation of BUDR (22), that is, into one DNA strand in each double helix.

It is also notable that BUDR exposure delayed until late in the BUDR susceptible period (Fig. 1) incompletely suppressed subsequent antibody production, not, apparently by partially damaging all of the antibody producing cells, but by eliminating most and sparing some. For the reduced antibody production followed a time curve very nearly parallel to the normal response, suggesting a diminished population of normal cells producing antibody. Histologically, cells containing antibody appeared normal but sparse. This is the result that would be expected if BUDR damaged only dividing cells and if some part of the cell-lines producing antibody had finished dividing before they were exposed to the analogue.

This interpretation of the results leads to several inferences about the events occurring in this anamnestic antibody response in tissue culture:

1. The development of the antibody production observed depends on cell multiplication during the 2nd, 3rd, and probably 4th days after antigen stimulation.

2. The progenitive cells that respond to the antigen stimulation are either resting or dividing at a relatively slow rate prior to the antigen stimulation and for approximately 1 day afterward.

In the experiment shown in Table V, the *in vitro* system was stimulated with one antigen and exposed to BUDR for 2.5 days. Then it was stimulated again with the same antigen and another antigen as well. It produced very little antibody to the first antigen but made a normal antibody response to the second. Our interpretation is that the progenitive cells stimulated early by the first antigen began to multiply, were damaged by BUDR, and were not available to respond to the repeat stimulation with that antigen; the progenitive cells which responded to the single late stimulation with the other antigen, however, had been unstimulated, non-dividing, and thus undamaged during the BUDR exposure. This suggests two more inferences about most, but not necessarily all, progenitive cells in the system:

1. The progenitive cells that respond to a specific antigen on a given day are the same cells what would have responded to a stimulus with that antigen 2.5 days earlier.

2. The progenitive cells that respond to one of these antigens and the progenitive cells that respond to the other antigen are, for the most part at least, different cells.

Dutton, Dutton, and Vaughan (23) hyperimmunized rabbits with ovalbumin. Two days after an anamnestic stimulus they prepared from the spleens cell suspensions which synthesized antiovalbumin *in vitro* for at least 48 hours. The rate of antibody synthesis usually increased nearly threefold during the first 24 hours *in vitro*, and BUDR exposure prevented this increase. Assuming that peak antibody production was not being approached prior to the anamnestic stimulation, this relatively small increase in rate and its suppression

suggests that conditions for multiplication may be poorer for the isolated cells of the suspension than for those in the node fragments.

The results and interpretations presented here for an *in vitro* system suggest that BUDR might also have a more general use for selectively damaging the multiplying members in a mammalian cell culture, analogous to the penicillin method for isolating bacterial mutants.

SUMMARY

Incorporation of 5-bromodeoxyuridine (BUDR) in the culture medium for the 2nd, 3rd, or 4th day after *in vitro* antigen stimulation of rabbit popliteal lymph node fragments suppressed the *in vitro* anamnestic antibody response described previously. Before or after this 3-day period, BUDR had no measurable effect. The results suggest that the antibody response in this *in vitro* system depends upon cell multiplication during this period.

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