INDUCTION AND REVERSAL OF IMMUNE PARALYSIS IN VITRO

BY V. S. BYERS,* PH.D., AND E. E. SERCARZ, PH.D.

(*From the Department of Bacteriology, University of California at Los Angeles, Los Angeles, California 90024*)

Do paralyzed cells exist? Or is paralysis induced by destruction of specific antigen-responsive cells? Recovery from paralysis has often been observed in vivo. Some of these reports indicate that antigen-responsive cells are paralyzed but can later recover upon removal of antigen (1, 2); others suggest that all such cells are killed during paralysis induction, and subsequent responses arise from newly arisen cells (3–6).

The question can be resolved with in vitro systems, in which paralysis induction is possible, but no new antigen-sensitive cells can be generated. In a previous paper, we have described the elements of such a model system for the induction of paralysis in vitro (7). If primed lymph node fragments are subjected to a high dose of antigen for 6 days, one can obliterate the typical response which would have been evoked by 2 hr exposure to antigen. Obliteration appears to support the concept of cell-killing; however since initiation of an in vitro response is almost impossible by the 6th day of culture, demonstration of reversible inhibition would also have been impossible. With shorter antigen exposure times, we can now reproducibly delay the appearance of a secondary antibody response in vitro.

This is a report of experiments which lead us to conclude that continued exposure to the appropriate antigen dose postpones the wave of response-dependent cell division which typically follows closely after optimal antigen stimulation. This is manifested by a delay in the onset of antibody formation.

Materials and Methods

Antigens and Drugs.—Diphtheria toxoid (DT)[1] was the generous gift of Mr. Leo Levine, Massachusetts State Antitoxin Laboratory, Jamaica Plains, Mass. Bovine serum albumin (BSA), five times recrystallized, was purchased from Pentex Biochemical, Kankakee, Ill. and

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; BUDR, 5-bromuracilde-oxyriboside; DT, diphtheria toxoid; I*BSA, ^{125}I bovine serum albumin.

from Armour Pharmaceutical Co., Chicago, Ill. BSA (Armour) and BSA (Pentex) were used interchangeably for primary in vivo injections, and for in vitro stimulations, with no noticeable differences. However BSA (Armour) was used for all assays. 5-bromuracildeoxyriboside (BUDR) was purchased from Calbiochem, Los Angeles, Calif.

Assays.—Passive hemagglutination: Media were titered by passive hemagglutination (8) in a microplate format (9). Titers are expressed in $\log_2$ dilution units; thus a titer of four indicates an end point dilution of 1:16. All are plotted as the arithmetic averages of the titers of one set of cultures, usually four per set.

Antigen-binding capacity: Media were also assayed for their ability to bind ^{125}I BSA (I*BSA) using the Farr method (10). K[^{125}I] was purchased from the New England Nuclear Corp., Boston, Mass., and BSA (Armour) was iodinated using the procedure of McConahey and Dixon (11). The diluent (BB-NRS) for both media and I*BSA was borate buffer, pH 8.4 supplemented with 10% normal rabbit serum (Pelfreez, Inc., Rogers, Ark.). Constant volumes of I*BSA and diluted media were incubated for 1 hr at 37°C. All reagents were cooled to 4°C, and ammonium sulfate was added to a final concentration of 50%. The precipitate obtained was washed once with 50% ammonium sulfate, resuspended in 0.5 ml buffer, and counted on a Nuclear-Chicago crystal scintillation detector (Autogamma). The percentage of total counts precipitated was calculated using the appropriate corrections, and expressed as micrograms of I*BSA bound per milliliter media.

Avidity: The avidity of antiserum or tissue culture media was estimated using the method described by Celada *et al.* (12) in which the volume (in microliters) of antiserum required to bind 50% of the available antigen is plotted against the total amount of antigen added. The resultant slopes are directly related to the avidity of the antibody. This volume was determined by the Farr method; four sets of each serial dilution were prepared, and a different concentration of I*BSA was added to each set in a constant volume.

*Lymph Node Culture.—*This organ culture system has been previously described (7). New Zealand white rabbits were given primary injections of 10 mg of BSA and 100 Lf DT in each rear foot pad. Popliteal lymph nodes were removed from two rabbits of the same age, which had been primed on the same day 2–10 months previously. The nodes were trimmed of fat, sectioned, and cut into 1 mm³ fragments in Petri dishes containing 4 ml sterile medium (minimal essential Eagle's medium supplemented with glutamine, hydrocortisone, penicillin, streptomycin, and 25% normal rabbit serum).

The appropriate antigen solution was added to dishes of randomly distributed fragments. These were incubated for 2 hr at 37°C in an atmosphere of 95% air-5% CO_2 (stimulation). The fragments in each dish were washed three times with a total of 15 ml of Hanks' balanced salt solution (Hanks'). 12 fragments were placed between layers of glass wool in each Leighton tube. 1 ml of medium was added to each and the stoppered tubes were incubated at 37°C in the CO_2-air atmosphere. Fresh medium was replaced at 1–4 day intervals, and the removed media were assayed for antibody content. Each value represents the amount of antibody produced by 1 culture (a tube of 12 fragments) during the interval between media changes. In the absence of other treatments, a "normal response" directed against the antigen used for stimulation was detectable in the culture fluid 5–6 days after culture.

*In Vitro Stimulation.—*Fragments were stimulated with either DT (5 Lf/ml), BSA (5 or 0.5 mg/ml), or with both BSA and DT. One culture could simultaneously produce both antibody responses. Therefore, an anti-DT response was usually induced in all cultures as a specificity control for inhibition of the anti-BSA response. In some cases, it was useful to stimulate fragments with one of the antigens on day 2 or 3 of culture, rather than on day 0 (delayed stimulation). This response would then begin on day 7 or 8 of culture, about 2 days after the normal response.

*Experimental Design.—*All experiments included sets of cultures treated as shown in Table I.

Medium containing 5 mg/ml BSA was added to one set (*BSA 0–3d*) immediately after preparation. 3 days later, this medium was removed, and each culture was washed six times with Hanks' (3 ml per wash); normal medium was replaced. It has been shown in a previous paper (7) that the amount of antigen remaining is insufficient to interfere with the antibody assays. Another set (stimulated) was incubated with 5 mg/ml BSA for 2 hr on day 0 (*BSA-2h*). Controls included two sets of stimulated cultures. One of these was not included in the 3 day wash, to check for inhibitory effects of the washing procedure. The other set, called "reconstruction" cultures, (*BSA 2h; 2–3d*) received 5 mg/ml BSA 1 or 2 days after stimulation. This antigen was removed by the 3 day wash, and the subsequent anti-BSA response was compared with a normal anti-BSA response to verify that antigen had been adequately removed from all cultures.

TABLE I

*Core Experiment**

Antigens‡	Exposure time, days after culture§	Anti-BSA response, code and symbol	Result
Experimental			
(a) BSA⎱ DT ⎰	2 hr, day 0	Stimulated BSA 2h	Normal response
(b) BSA	First 3 days (days 0–3)	Postponed BSA 0–3d	Delayed anti-BSA response
DT	2 hr, day 0		Normal anti-DT response
Controls			Control for:
(c) No antigen		Unstimulated	Unstimulated response
(d) BSA⎱ DT ⎰	2 hr, day 0; BSA days 2–3	Reconstruction BSA 2h; 2–3d	Residual antigen masking of antibody production
(e) BSA⎱ DT ⎰	2 hr, day 0*		Inhibitory effect of wash

* All sets were washed on day 3 except set (e).

‡ Antigen concentrations: 5 mg/ml BSA and 5 Lf/ml DT.

§ Day 0 of culture is the day on which the nodes were removed and cultured.

RESULTS

Postponement of Normal Responses.—A normal antibody response was produced by exposing primed lymph node fragments to BSA for 2 hr (*BSA 2h*) immediately after they were prepared (day 0 of culture). If instead, the fragments remained in an antigen-rich environment for the first 3 days of culture (*BSA 0-3d*) the additional period of exposure caused the onset of the response to be postponed and shifted the entire curve to the right. (Fig. 1). Comparison of the two curves strongly suggested that induction was reversibly inhibited by a high antigen concentration of 5 mg/ml BSA.

Requirements for Postponement.—The postponed response required both pro-

longed antigen exposure and high antigen concentrations. 2-hr incubation with either 5 mg/ml BSA or 0.5 mg/ml BSA resulted in a normal response. Likewise, the response of cultures exposed to 0.5 mg/ml BSA or 0.05 mg/ml BSA during the first 3 days was normal.
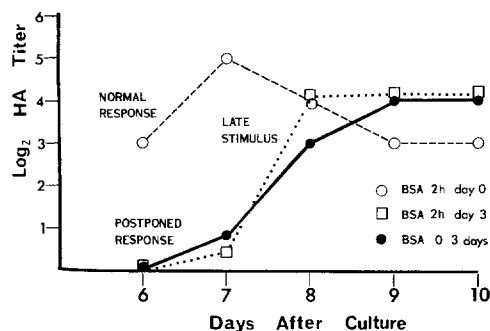

FIG. 1. Normal *vs.* postponed response. Anti-BSA responses of lymph node cultures exposed to 5 mg/ml BSA in vitro. Antigen was added to two sets of these cultures immediately after preparation (day 0 of culture) and either removed 2 hr later (O-----O) or 3 days later (●——●). Antigen was added to the third set (□····□) on the 3rd day of culture and removed 2 hr later. Daily media changes were assayed by passive hemagglutination.
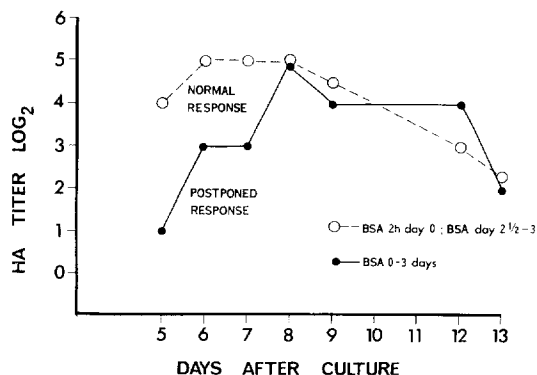

FIG. 2. Wash control. Anti-BSA responses of cultures after 5 mg/ml BSA was washed out on day 3 of culture. BSA was added to both sets of cultures on day 0. It was removed from one set (O-----O) 2 hr later, then readded shortly before the 3-day wash (*BSA 2h; 2½-3d*). It was not removed from the other set (●——●) until the 3-day wash (*BSA 0-3d*).

Wash Controls. Each experiment included sets of cultures treated as shown in Table I. All were washed with Hanks' 3 days after culture. This removed antigen from *BSA 0-3d* cultures, but had no inhibitory effect on the response, since neither the anti-DT response (of *BSA 0-3d* cultures) nor the normal anti-BSA response was reduced in titer. Normal and "reconstruction" responses

were compared and found to be identical in almost all experiments. This demonstrated that the wash effectively removed all the antigen which would have been capable of masking the antibody and concealing the initial phase of the response.

Reproducibility.—In the series of 24 experiments reported here, BSA *0-3d* treatment invariably delayed the onset of antibody formation. This delay usually lasted about 2 days. Although the entire response curve was often shifted to the right, this was not predictable. Sometimes the duration of antibody formation was not prolonged (Fig. 2); in other experiments, the entire
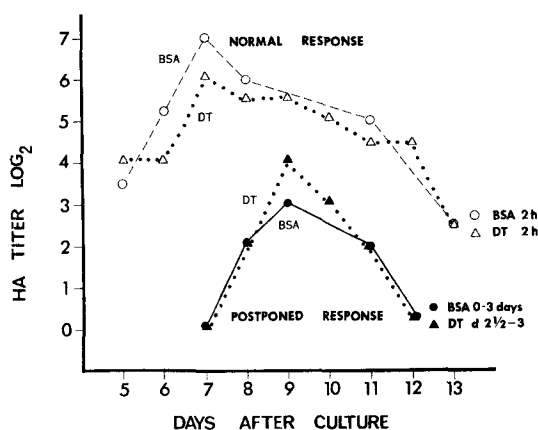

FIG. 3. Postponed response *vs.* delayed stimulus. The top two curves represent the anti-BSA response (O-----O) or the anti-DT response (△·····△) of cultures exposed to both antigens for 2 hr on day 0. The lower two curves indicate the anti-BSA response of cultures exposed to 5 mg/ml BSA for the first 3 days of culture (●——●) or the anti-DT response of cultures first exposed to DT for 12 hr on day 2½ of culture. (▲·····▲).

response would be shifted, but with severely decreased titers (Fig. 3). Several of these experiments included cultures stimulated with DT on day 2, rather than on day 0. When the resulting anti-DT titers were abnormally low, the postponed anti-BSA response in the experiment was also quite low (Fig. 3), suggesting that weak postponed responses were related to premature loss of general "stimulability" in some experiments.

Reversible Paralysis.—If this phenomenological delay in the response truly reflected a short-term, reversible inhibition of some step(s) necessary for immune induction ("reversible paralysis"), we felt that the following criteria had to be met: (*a*) the same cell population that produced the normal response should be responsible for the postponed response; (*b*) a delay in some biochemical or maturation event preparatory to antibody synthesis should be correlated with the delay in antibody production.
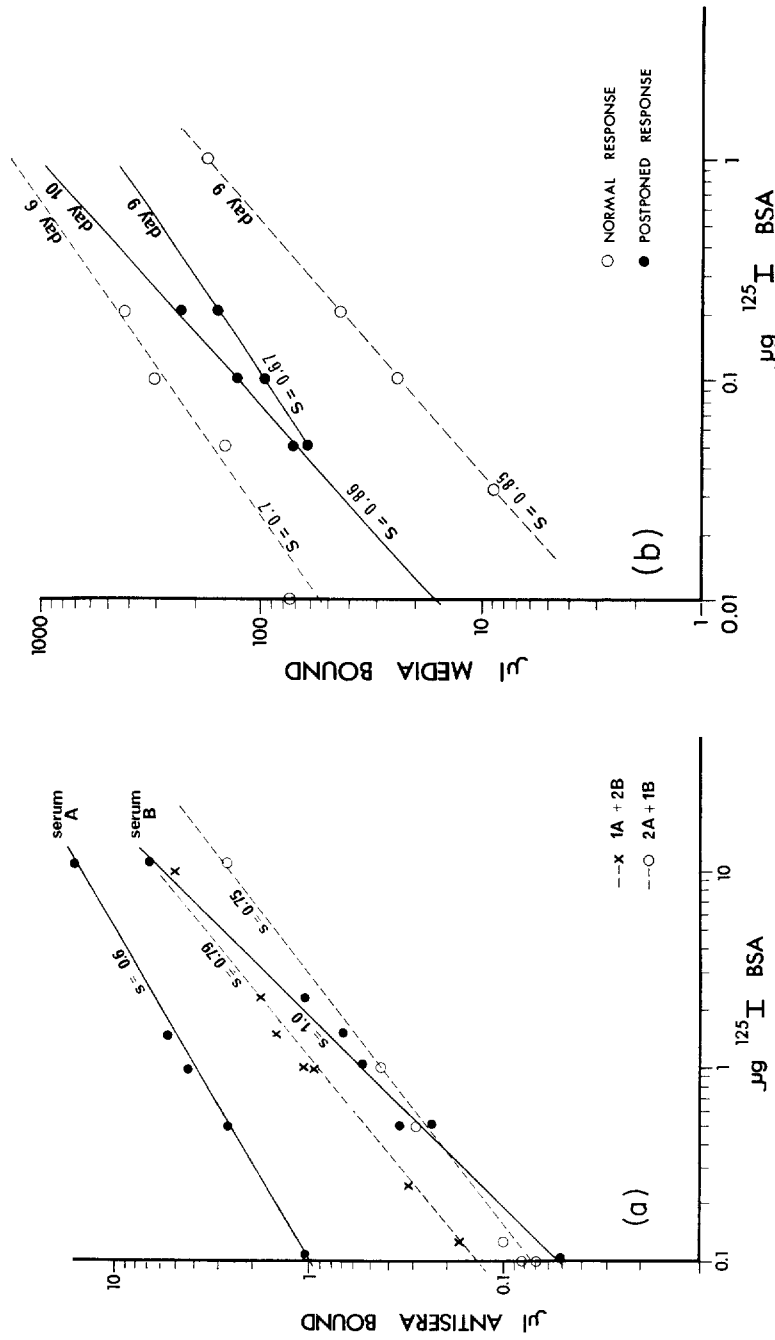
Fig. 4. Avidity assays. *a*. Serum. The avidity of antiserum A, taken from a rabbit 22 days after primary BSA injection, and that of antiserum B, obtained from a rabbit hyperimmune to BSA is indicated by the slope of the lines. Mixtures comprised of two parts serum A and 1 part serum B (○----○) or one part serum A and two parts serum B (×----×) were also assayed. *b*. Media. The avidities of media removed at various times during the postponed response (——●) or during the normal response (○----○) are measured by the slopes of their lines.

(a) *Avidity of normal and postponed responses:* It has been suggested (13) that paralyzing antigen preferentially kills cells with high affinity receptors, and any antibody produced by the surviving cells is of low affinity. We therefore explored the avidity of normal and postponed responses to determine whether there was a gross difference between the affinity characteristics of the cell populations.

The avidity assay method was patterned after that developed by Celada *et al.* (see Materials and Methods). The amount of removed culture media which bound 50% of the available I*BSA was determined at various antigen concentrations, and the results plotted on the coordinates of Fig. 4. If the volume of medium binding by 50% of I*BSA was directly proportional to the antigen concentration, the resultant line would have a slope of 1.0, classifying the avidity as maximal. If, however, this volume were disproportionately decreased by increasing antigen concentrations, the slope would be less than 1.0, indicating that the avidity of the sample was lower.

Fig. 4 *a* illustrates a pilot experiment, showing that the assay did not preferentially detect antibody of high affinity. Two reference antisera were assayed separately (solid lines), and then mixed in two different ratios (dotted lines). "High avidity" serum A was obtained from a hyperimmune BSA rabbit; "medium avidity" serum B was taken from a rabbit 22 days after primary BSA injection. Both mixtures of these sera yielded lines with slopes of intermediate values. This demonstrated that even the addition of moderately avid serum lowered the avidity value of the sample.

The avidity of anti-BSA antibodies produced during the normal and postponed responses were then compared (Fig. 4 *b*). Culture media taken at various times from both sets of cultures were assayed, and it was found that the avidity of both responses was substantially identical. This result supports the view that the postponed response arises from the same population of cells as the normal response, and not from a selected, antigen-resistant subpopulation.

(b) *Postponement of cell proliferation:* Postponement of antibody production might reflect the temporary inhibition of any or all phases of the intricate induction period. Since a wave of cell proliferation follows soon after antigen stimulation, DNA synthesis is one of the earliest biochemical events characteristic of induction (14, 15). If an antigen-laden environment blocks an inductive event(s) critical for cell-division, induction would await antigen removal, and both the onset and the completion of this wave of cell proliferation would be postponed in *BSA 0-3d* cultures.

Cells which divide in the presence of BUDR incorporate it, thereby committing suicide. Therefore this drug can be used to mark the days on which specific precursor cells are dividing. BUDR pulses during this time will eliminate the subsequent response. Groups of fragments were either treated with *BSA 2h* (normal response) or with *BSA 0-3d* (postponed response). Four cultures from

(a) DELAYED ONSET OF ANTIBODY PRODUCTION
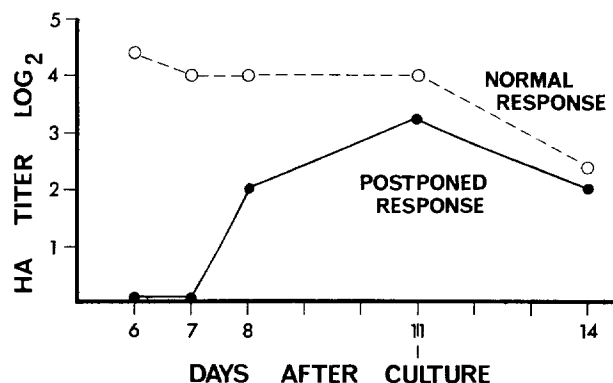
(b) DELAYED BUDR SENSITIVITY

	BSA 2h day 0	BSA 0-3 days
	µg/ml	µg/ml
No BUDR	0.69	0.50
BUDR, days :		
0-1	0.61	0.53
1-2	0.38	0.50
2-3	0.21	0.64
3-4	0.14	0.28
4-5	0.61	0.11

Fig. 5. BUDR sensitivity of normal vs. postponed responses. a. Drug-free normal and postponed responses. Anti-BSA response of cultures exposed to 5 mg/ml BSA during the first 2 hr (O-----O) or during the first 3 days (●——●) of culture, assayed by passive hemagglutination. b. Effect of BUDR pulses on normal and postponed responses. BUDR was added to four cultures from both of these groups during one of the periods indicated. The antibody binding capacity of media removed during days 8–11 of culture were assayed, and the average values are expressed as micrograms I*BSA bound per milliliter of media. The top line shows the amount bound by drug-free controls.

each group were exposed to 1-day BUDR pulses during one of the first 5 or 6 days preceding antibody production. Their antibody production from days 8–11 of culture was measured by antigen-binding assays (Fig. 5 b). The sensitivity pattern of the normal response (left column) indicated that no division occurs

during the 1st day after stimulation, but that almost all response-precursor cells are engaged in division during the 2nd, 3rd, and 4th days. After the 4th day, the replicative events among these cells are complete. This pattern was compared with that found in *BSA 0-3d* cultures. It was noted that such cultures required additional time to complete the requisite division events. Thus, a BUDR pulse 5 days after initial antigen contact (days 4–5 of culture) although
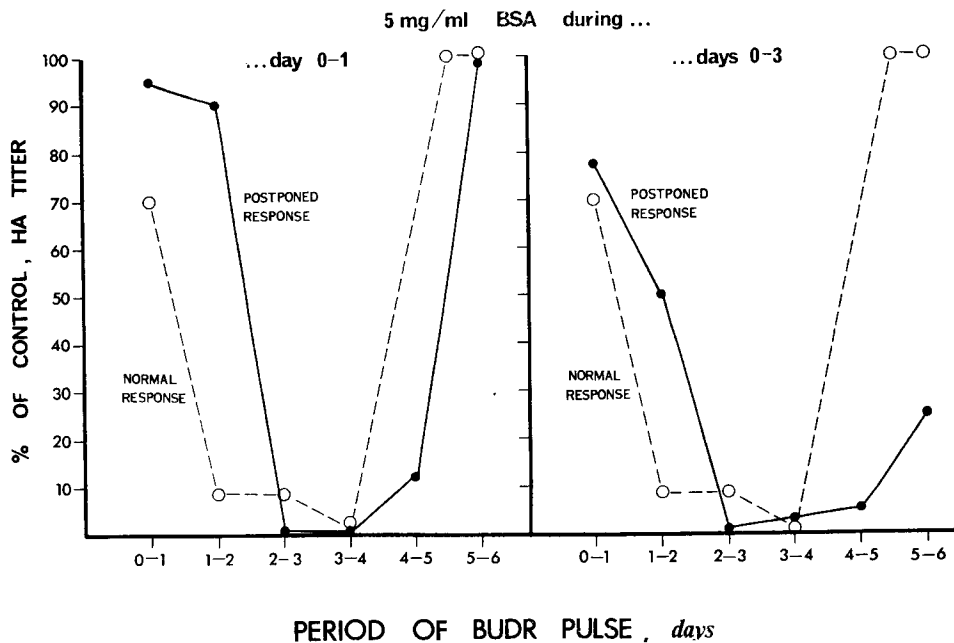

FIG. 6. BUDR inhibition of cultures exposed to BSA. Three groups of cultures were exposed to 5 mg/ml BSA on day 0 for: two hr (O-----O), 1 day, Fig. 6 Left (●———●), or 3 days, Fig. 6 right (●———●). BUDR was added to four cultures in each group during the 1-day periods indicated. The media removed on day 9 of culture were assayed by passive hemagglutination. The titers of BUDR-treated cultures were compared with those of drug-free controls, which were normalized to give 100% response. The per cent inhibition is plotted.

never inhibitory to the normal response, almost eliminated the postponed response. This was matched by the corollary finding that cell division began later than usual. A BUDR pulse given 1 or 2 days after initial antigen contact abolished the normal response, but the postponed response of *BSA 0-3d* cultures was unaffected. By preventing cell division, prolonged antigen exposure actually "protected" the cultures from the damaging effects of BUDR. Such protection by paralyzing antigen indicates that it delayed the onset of cell proliferation.

In a few experiments, especially those in which low antibody titers were pro-
duced during the postponed response, this protection effect was not obvious.
To specifically focus on the *onset* of proliferation, we therefore shortened the
period of BSA exposure to 1 day. Fig. 6 compares the inhibitory effect of BUDR
pulses on cultures which were treated with *BSA 2h*, *BSA 0-1d*, or *BSA 0-3d*.
Inhibitory effects of these 1-day pulses of BUDR are presented near the plateau
of both normal and postponed responses. It was found that protection from the
early killing effect of BUDR was pronounced when cultures were exposed to
BSA for only the 1st day of culture (see Fig. 6, left); furthermore the response
remained sensitive to BUDR for 1 additional day. This prolongation of BUDR
sensitivity was even more evident in *BSA 0-3d* cultures (see Fig. 6, right)
where precursor cell division occurred as late as day 5-6 of culture.

Both types of experiments support the concept that prolonged exposure to
high antigen concentrations delays one of the very early biochemical events
which prepares for antibody production, DNA synthesis. As a result, the onset
of antibody formation is postponed.

DISCUSSION

The signal for immune induction is activated by antigen removal. This signal
can be postponed for as long as 2 or 3 days by incubating cultures of primed
rabbit lymph nodes with a high level of antigen for 3 days rather than 2 hr.
Consequently, the immune response is also postponed. Since prevention of in-
duction in an environment of supraoptimal antigen fulfills all criteria for im-
mune paralysis, these experiments demonstrate that paralysis is reversible. We
conclude that paralyzed cells do exist, and that removal of antigen reverses
their unresponsive state. Therefore, paralysis induction does not necessarily
require the death of responsive cells.

Reversible paralysis was demonstrated by two criteria. (*a*) DNA synthesis
and cell proliferation, a critical phase of immune induction, occurred later than
usual, and (*b*) the cell population responding after temporary paralysis had the
same affinity characteristics as the population that produced a normal response.

BUDR was used to indicate the days on which the population of response-
dependent precursor cells were dividing. Normally this wave of cell division
began 1 day after brief antigen contact, and it ended well before antibody pro-
duction commenced. If antigen contact was prolonged for 24 hr, the onset of
cell division was also delayed by an additional day, and instead began 2 days
after antigen exposure. Similarly, when antigen exposure was continued for
3 days, cell division was concommitantly later in onset and completion. By
temporarily preventing cell division, paralyzing antigen initially protected the
cultures from BUDR killing, but they were then susceptible to the drug later
than usual. Thus, the schedule for cell proliferation could be forced to follow a

new time pattern which was correlated with the time of antigen removal, and not with the time of initial antigen contact.

Presumably cell division is just one of a series of events preceding antibody formation which can be delayed in the presence of supraoptimal antigen concentrations. Ivanyi et. al. (16) also studied in vitro paralysis reversal, and demonstrated that delayed antibody production was accompanied by a delay in another of the early biochemical events of induction, RNA synthesis. An apparent case of paralysis reversal was studied by Scott and Waksman (17). Paralysis was induced by large amounts of bovine gamma globulin. The antibody response which was subsequently noted was shown to be directed against a contaminant in the antigen preparation. For this reason we regularly used BSA (Armour) which is over 99% pure, for all assays and demonstrated that antibodies produced during the postponed response were capable of binding almost 100% of the available I*BSA.

The fate of a paralyzed cell has been controversial. These experiments provide evidence that such cells are not killed or permanently inactivated. New antigen-responsive cells cannot be generated in vitro in this culture system (18). Therefore if paralysis were achieved by the permanent inactivation of a majority of the pool of responsive cells, the postponed response would have to be attributed to a small minority of antigen-resistant survivors, stimulated rather than paralyzed by antigen, probably because of the low affinity of their receptors. The critical point is that the induction period in this subpopulation of antigen-resistant cells would be expected to commence according to a normal time table. This is not the case. Moreover, the avidity of the response should be markedly lower than normal. This also is not found. Indeed the avidity values of both responses were nearly identical, supportive evidence that the same population of cells produced both the normal and the postponed responses.

Cultures exposed to BSA for 3 days began antibody production 1 or 2 days later than usual, not 3 days later. This suggests that even in the presence of high amounts of antigen some of the inductive events can take place. Antigen removal must then permit those reactions which complete the induction process. In another report (19), a hypothetical model was presented, describing the occurrence and order of events during antigen exposure. Cell finding prior to interaction was proposed as one event which might take place in an antigen-laden environment.

It was suggested years ago that paralysis usually precedes immunity in the normal course of the immune response (20, 21). This theory is not only supported by the evidence presented here, but also by the experiments of Steiner and Eisen (22), who reported that the characteristic increase in antibody affinity which follows injection of moderate amounts of antigen is delayed by the injection of large amounts of antigen. The population of cells that respond to

antigen stimulation probably represents the entire spectrum of antibody affinity. When large antigen doses are injected, those with low-affinity receptors would produce antibody first since they would be less easily paralyzed; high-affinity antibody would only be found later in the response, after the initial paralysis of cells with high-affinity receptors had been reversed. Such a view suggests that temporary paralysis is extremely common.

The reversible paralysis which was studied in these experiments is of short duration. It is quite possible that this is the early phase of the classically studied long-term paralysis. If paralyzing antigen remains bound to their receptors for a sufficient period, these inhibited cells might be judged senile, and purged. Such a system would insure a lack of response to self-determinants. At the present, alternative suggestions involving an acute antigen-induced killing have no direct experimental basis.

SUMMARY

Induction of the immune response can only be completed after antigen is removed from the cellular environment. Primed rabbit lymph node fragments were cultured in vitro with 5 mg/ml BSA. If antigen was removed from the fragments 2 hr later, they produced a normal anti-BSA response, which was first evident 5 days later. If antigen removal was delayed for 3 days, the onset of the response was postponed for 2 to 3 days.

Pulses with BUDR marked the periods of cell proliferation in both sets of cultures, and established that the postponement of antibody production was preceded by a postponement in the wave of proliferation among precursors of antibody forming cells.

The similarity in avidity of antibody-containing fluids from normal and postponed cultures support the idea that the same cell population produced the response in each case.

It was concluded that a reversible state of paralysis could be instituted in antigen-responsive cells, and this state did not depend upon cell-killing. The widespread incidence of temporary paralysis as an early aspect of the immune response was discussed.

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