THE X-Y-Z SCHEME OF IMMUNOCYTE MATURATION

V. Paralysis of Memory Cells

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It has been well documented that the sustained injection of large doses of antigen will render an animal incapable of responding to further antigenic challenge. This has been observed in both neonatal and adult animals; in both cases, the inhibition is assumed to operate on the level of the virgin, competent (X) cells.

Investigators have attempted to determine if the induction of immune unresponsiveness is also possible in immunologically experienced animals (1–14). This is an interesting question because of the large body of evidence indicating that one of the cell types responding to a second antigen exposure, termed a Y or memory cell, is qualitatively different from the X cells which respond to a first antigen exposure (15). This memory cell concept is useful in explaining the rapid onset of proliferation and antibody synthesis characteristic of a secondary response.

Several workers, using both in vitro and in vivo culture systems, have studied the dose-responsiveness of transferred primed cells to antigen (5, 8, 10, 13); generally, as the stimulatory antigen concentration is increased, the magnitude of the secondary response reaches a plateau, and then begins to decrease. In many of these experiments, the reduced response was not due to masking of the antibody produced by an excess of antigen; thus hyporesponsiveness of primed cells had been induced. Brent and Gowland (2) used a skin graft system in mice to show that presensitized mice could be paralyzed as easily as nonsensitized mice. Siskind and Howard (12), using pneumococcal polysaccharide type II as an antigen, reported that preimmunized mice were slightly more susceptible to paralysis induction than normal animals. Mäkelä and Mitchison (8) noted that as the concentration of bovine serum albumin (BSA) used to challenge immunized mouse lymphoid cells in vitro was increased, within the range of 10–100 mg, the response was dramatically reduced as assayed by antibody titers; iododeoxyuridine (IUdR) uptake, as a criterion of cell proliferation, was also inhibited. Makinodan et al. (10) placed Millipore chambers containing immunized mouse spleen cells into X-irradiated recipients, and determined that a high dose of the antigen used for priming (either sheep red cells or BSA) would paralyze these cells, suppressing both antibody formation and cellular proliferation.

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Several experiments dealing with this problem have involved prolonged cellular contact with antigen (1, 4, 7, 9–12), allowing two possible explanations for the subsequent unresponsive state: (*a*) the X and Y cell populations are paralyzed, without initial antibody formation, or (*b*) the X cell population is paralyzed, but the Y cell population is "used up" or exhausted. The inactive state following the initial memory cell response, would be a manifestation of this X cell paralysis. It has been shown in a previous paper (16) that the injection of a large amount of antigen into an immunized animal does result in an initial production of large amounts of antibody, followed by an unresponsive state.

We have attempted to ascertain whether real paralysis of the memory cell is possible. We used primed rabbit popliteal lymph node fragments in a tissue culture system (17) several features of which are well suited for exploration of this problem: (*a*) As documented in a previous paper (16), a primary response to BSA cannot be induced in this system; hence the response under observation is restricted solely to that of the memory cells. (*b*) The antibody titers do not reach a peak until 9–12 days after in vitro induction. Therefore the tissues can be exposed to large amounts of antigen during the early days of culture without risk of the excess antigen masking the normal response. (*c*) The antigen concentration and the duration of antigen exposure can be carefully controlled. We found that the values of these two variables can be adjusted in such a manner as to allow no detectable antibody production by the primed node fragments.

Materials and Methods

Biologicals.—New Zealand white rabbits were primed with 10 mg BSA and either 100 Lf diphtheria toxoid or 2.3 mg keyhole limpet hemocyanin (KLH) in each rear footpad. BSA, five times recrystallized, was obtained from Pentex, Inc., Kankakee, Ill. KLH was prepared by the method of Campbell et al. (18). The keyhole limpets were purchased from Pacific Biomarine Supply Co., Venice, Calif. Diphtheria toxoid was the gift of Leo Levine, Massachusetts State Antitoxin Labs, Jamaica Plains, Mass.

The medium used in all experiments was minimal Eagle's medium, obtained from Hyland Laboratories, Los Angeles, Calif., and supplemented with 300 μg/ml L-glutamine, 1 μM hydrocortisone hemisuccinate, 60 units/ml penicillin G, 58 μg/ml streptomycin, and 25% normal rabbit serum (purchased from Pelfreez Inc., Rogers, Ark.). It was sterilized by filtration through washed Millipore filters. When necessary, this medium was supplemented with different concentrations of filter-sterilized antigen.

Tissue Culture.—2–10 months after priming, the rabbits were killed and the popliteal lymph nodes (which drain the footpads) were removed, trimmed of fat, and cut into 1 mm^3 fragments in a Petri dish containing culture medium. These dishes were placed in an incubator containing a mixture of 5% CO_2–95% air for approximately 2 hr. If stimulation of the fragments was desired, antigen was added to this medium at a concentration of 0.5 mg/ml (unless otherwise stated). After the 2 hr incubation, the antigen was removed and the fragments washed three times with 15 ml Hanks' balanced salt solution (Hanks' purchased from Hyland Labs). Tubes containing such fragments were called "stimulated tubes."

12 fragments were placed in each Leighton tube, blanketed with glass wool (19), coveres with 1 ml medium, and incubated at 37°C in the CO_2-air mixture. The culture medium wad replaced at 1–4 day intervals, and the removed media, called "used media," were assayed for

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antibody content. Hence, the values reported refer to the antibody produced by the fragments in one tube during the period that the used medium was left in the tube.

To wash the fragments in tubes which had contained antigen for several days, the media were removed, and 2.5 ml Hanks' solution was added to each tube, and removed after about 5 min. This was repeated five additional times, after which 1 ml of antigen-free medium was replaced. This special washing procedure is called "wash-out." If contamination occurred in any tube, it was discarded and its previous titer disregarded.

Assays.—

Hemagglutination: The used media were titered by passive hemagglutination in a microplate format (20, 21). Titers are expressed in $\log_2$ dilution units (thus an end point of 1:4 dilution has a titer of 2). The titers obtained by this method were uniformly 4–5 tubes lower than the macrotiter method in tubes (20), but the former method was used for convenience and clarity of end point.

Antigen-binding capacity: Used media were also assayed for their ability to bind ^{125}I-BSA (I*BSA) using the Farr technique (22). K^{125}I was purchased from the New England Nuclear Corp., Boston, Mass., and the BSA was iodinated using the procedure of McConahey and Dixon (23). The assays were carried out in two ways. In the first, used medium was incubated with I*BSA for 1 hr, after which borate buffer, pH 8.4, was added to the mixture. All reagents were cooled to 4°C, and ammonium sulfate was added to a final concentration of 50%. The precipitate obtained was washed one time with 50% ammonium sulfate and counted on a Baird-Atomic crystal scintillation detector. The percentage of the total counts precipitated was calculated, with appropriate corrections. More often however, a method termed the "modified Farr" was used. This involved the addition of I*BSA to the fresh medium before it was placed on selected tubes. Thus the I*BSA could bind to the antibody as soon as it was produced. The used medium was then precipitated with a final concentration of 50% ammonium sulfate as previously described and the percentage of I*BSA bound was calculated. This latter assay was found to be more sensitive for our purposes than the regular Farr assay.

RESULTS

Inhibition of the Secondary Response with 5 mg/ml BSA.—Exposure of the primed node fragments to 0.5 mg/ml BSA for the first 2 hr after preparation resulted in an antibody response which began about 5 days after culture, peaked on days 9–12, and continued for about 1 additional wk, with the titers steadily declining. Such tubes, called "stimulated" were included in each experiment to serve as positive controls. Likewise, unstimulated tubes were also included as negative controls. Occasionally, a low titer was produced by some of these tubes; such an unstimulated response is seen in many tissue culture systems of this type, and is believed to result from sequestered antigen released during the fragmentation of the lymph node.

Increasing the concentration of BSA during the 2 hr exposure period to as much as 5 mg/ml still did not inhibit the response, but slightly augmented it. Therefore, different concentrations of antigen were added to the medium for the first 6 days of culture, "washed-out" as described in the Materials and Methods section, and antigen-free medium was replaced. If the antigen concentration during the 6 day exposure was 0.05 mg/ml BSA, the used media removed from these tubes subsequent to wash out of antigen (day 7–9 of

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culture) had antibody levels almost equal in titer to the positive controls. 6-day hemagglutination titers were negative, due to masking by the excess BSA present on day 6 of culture. The same results were obtained even when the BSA concentration was increased to 1 mg/ml. However, inhibition was achieved in tubes exposed to 5 mg/ml BSA for the first 6 days of culture; these fragments usually produced no antibody for the duration of the experiment (Fig. 1).

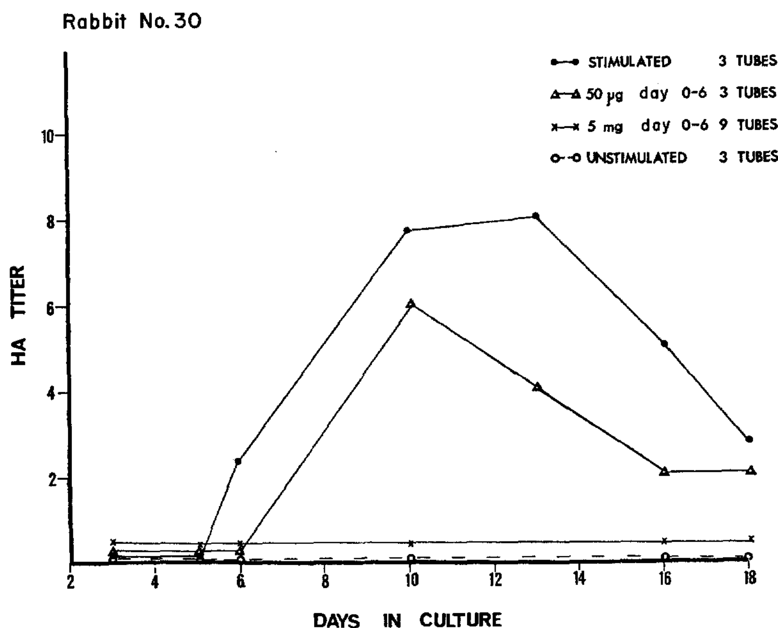

FIG. 1. Anti-BSA response inhibition by 5 mg/ml BSA from days 0 to 6. Fragments were either stimulated immediately after preparation by incubation for 2 hr with 0.5 mg/ml BSA (●——●); not stimulated (○-----○); or incubated with 0.05 mg/ml BSA (△——△) or 5 mg/ml BSA (x——x) for the first 6 days of culture. Each point represents antibody formed since the last change of medium. The ordinate units are reciprocals of the $\log_2$ dilution.

Effectiveness of the Wash-Out Procedure.—Several experiments were performed to prove that the inhibition was not an artifact due to antigen masking.

*1. Wash-out of I*BSA:* Fragments were exposed 5 to mg/ml I*BSA for the first 6 days of culture, after which the tubes were washed-out. This wash-out was monitored by counting each of the six washes, and the residual tube radioactivity at the end of the procedure. An amount of radioactivity equivalent to 2 μg/ml BSA (or 0.04%) remained in the tube, although the last three washes contained no radioactive antigen. To determine whether this residual nonelutable antigen could combine with antibody formed by the

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TABLE I
Wash-Out Reconstruction Using Modified Farr Assay

A. Intrinsic Antibody Produced by Primed Node Fragments

Day 0	Prepared 9 stimulated tubes and 9 unstimulated tubes
Day 5½	Add antigen-containing media to 6 tubes in each set: 5 mg BSA (3 tubes), 50 μg BSA (3 tubes), no BSA (3 tubes); 5 mg BSA (3 tubes), 50 μg BSA (3 tubes), no BSA (3 tubes)
Day 6	Wash out all tubes; replace medium supplemented with I*BSA, 0.2 μg/ml
Day 8	Remove used I*BSA media; assay for percent I*BSA bound to intrinsic antibody by precipitation with 50% ammonium sulfate:

	Stimulated tubes				Unstimulated tubes			
	%	%	%	avg %	%	%	%	avg %
5 mg	24	34	44	34	6	3	5	5
50 μg	38	34	27	33	9	6	6	7
None	38	22	39	33	18	10	14	14

B. Extrinsic Antibody Added to Unprimed Node Fragments

Day 0	Prepare 5 tubes using fragments from a normal node. Add antigen-containing media to 3 of these tubes: 5 mg BSA (3 tubes); No BSA (2 tubes)
Day 2	Wash out all tubes; add I*BSA medium, 0.2 μg/ml
Day 3	Add a total of 0.1 ml anti-BSA to each tube over a 6-hr period
Day 4	Remove media, assay for per cent of I*BSA bound to extrinsic antibody:

	%	%	%	avg %
5 mg BSA	55	52	63	57
No BSA	56	66		61

fragments and present in the medium, the following reconstruction experiment was performed.

2. Reconstruction using the modified Farr assay: Low-level antibody production is detectable by day 7 of culture. Therefore, on day 5½, medium containing either 0.05 mg/ml BSA or 5 mg/ml BSA was added to stimulated or unstimulated tubes for ½ day. On the 6th day, both sets of tubes were washed-out as usual, and normal medium supplemented with I*BSA was added to each tube. One day later, these used media were assayed for the

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presence of I*BSA bound to antibody produced during day 6–7. If there had been a significant amount of BSA left in the washed tube, it should have competed with the I*BSA to reduce the percentage of I*BSA bound to antibody. Three reference tubes from each set were given no antigen on day 5½–6. None of these media removed from stimulated tubes showed a reduction

TABLE II

Wash-Out Reconstruction Assayed by Hemagglutination

Rabbit No.	Stimulated*	Period of exposure to 5mg/ml BSA‡	Titer§										No. of tubes responding
			Days after culture										
			6	7	8	9	10	11	13	14	16	18	
1-91	Yes	None		2.5			7			5.3			6/6
		Days 3–6		0			6.5			6			6/6
1-72	Yes	None		3.8		4.5			6.3			7	4/4
	Yes	Days 3–6		0		4			6.5			6.3	4/4
2-50	Yes	None	0.8					4.1		4.5		4.5	17/20
	Yes	Days 1½–6	—		1.5			4		4.5		3.7	4/6
	Yes	Days 1–6	—		0.5			3.8		4.2		4.5	4/6
2-34	Yes	2 hr–6 day (added immediately after stimulation)					4		2.8		3		4/6
	Yes	Day ½–6					2.8		3		2.5		4/5
	Yes	Day 1–6					3		3		—		6/6
	Yes	None			5.5		5.4		1.8		2.5		9/9
	No	Day 0–6 (typical inhibitory treatment)					2.5		3		—		2/16

* Fragments were stimulated with 500 μg/ml BSA for the first 2 hr after preparation and placed in tubes.

‡ 5 mg/ml BSA-medium was added during the intervals shown.

§ The average titers of the responding tubes are presented as the reciprocal of the $\log_2$ dilution.

in the amount of I*BSA bound (Table IA) although the antigen treatment of the unstimulated fragments caused a slight reduction of binding. The insignificant isotope dilution in this experiment indicates that although there may be a small amount of BSA remaining in the 5 mg/ml BSA 0–6 day tubes immediately after wash-out, it is unable to mask any appreciable response. Table IB illustrates a similar reconstruction experiment using unprimed nodes and extrinsic antibody; again the binding of labeled antigen was not reduced by residual unlabeled BSA.

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3. Reconstruction experiments assayed by hemagglutination: Medium containing 5 mg/ml BSA was added to stimulated tubes at various times between days 1–6 after culture. On day 6 these tubes were washed-out and antigen-free medium was replaced. Assay of the first change of used media showed titers slightly lower than the positive controls, indicating that this assay is more sensitive to residual antigen than the modified Farr assay. However, one medium change later, all tubes yielded equivalent titers (Table II). When the high concentration of BSA was added within the 1st day of culture, some stimulated tubes produced no antibody titers, but in the majority of tubes the titers were either normal or slightly lowered. Even if the inhibitory treatment was begun immediately after stimulation, a majority of the tubes still responded. Apparently, once adequately induced, there is an irreversible commitment to antibody formation.

Test of Initial Antibody Production by Inhibited Tubes.—Secondary unresponsiveness produced by high antigen doses may be divided into two categories: *exhaustion* is characterized by initial antibody production followed by an unresponsive state; in *paralysis* the response never occurs. To distinguish between these two, the modified Farr test was utilized, for although we had demonstrated a lack of response in the 5 mg/ml BSA 0–6 day tubes following wash-out, the possibility remained that an early, short-lived antibody response had occurred. Therefore, at different times during the first 6 days of culture, tubes containing antigen were washed-out in the usual manner, and medium containing 0.2 μg/ml I*BSA was added. After 1 or 2 days, these used media were assayed for I*BSA bound by antibody produced during this time. Table III illustrates that slight antibody formation had begun as early as day 4, both in the stimulated tubes, and in those containing 0.05 mg/ml BSA. In contrast, those tubes receiving the inhibitory amount of antigen (5 mg/ml) showed less binding than the unstimulated controls. We took the lack of detectable antibody formation at any time during the culture period as evidence of paralysis rather than exhaustion.

Also shown in Table III are media from the same experiment, assayed by the usual Farr method, illustrating that the modified Farr is the more sensitive of the two assays.

Specificity of Inhibition.—Keyhole limpet hemocyanin (KLH), when used as a priming agent, results in a long-lasting antibody response which can be continued in vitro. Therefore, nodes which had been primed with both BSA and KLH were cultured. Although 5 mg/ml BSA 0–6 day treatment resulted in the usual anti-BSA inhibition, the anti-KLH response continued unabated in all tubes. This demonstrated that the BSA was neither toxic, nor nonspecifically inhibitory. In another experiment, 5 mg/ml of human serum albumin, an antigen slightly cross-reactive with BSA (24) was added to BSA-stimulated tubes for the first 6 days of culture. On the 9th day, anti-BSA titers were approximately equal to those given by the reference stimulated

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TABLE III

Assay of Early and Late Antibody Formation

Treatment	Per cent I*BSA bound									
	Modified Farr*						Standard Farr on used media‡			
	Day of exposure to 0.2 μg/ml ¹²⁵I BSA						Day of exposure of used media			
	2–3	3–4	5–6	6–7	9–10	9–11	6–7	7–8	8–9	9–10
Stimulated	—	4	16.5	31.0	27.0	60.0	27	27	24	23
Unstimulated	—	3.0	4.0	11.7	17.6	16.9	7.3	6.0	3.3	3.3
50 μg/ml BSA										
days: 0–2	1.5	—	—	—	—	—	—	—	—	—
0–3	—	2.3	—	—	—	—	—	—	—	—
0–5	—	—	2.0	—	—	—	—	—	—	—
0–6	—	—	—	26.5	—	49.3	—	8.5	21.6	28
5 mg/ml BSA										
days: 0–2	3.5	—	—	—	—	—	—	—	—	—
0–3	—	1.3	—	—	—	—	—	—	—	—
0–6				8.7		6		3.5	3	3

* Each value is the average of 2–6 tubes. After the test, tubes were discarded.

‡ 0.2 μg/ml I*BSA was used.

TABLE IV

Inhibition Summary

Rabbit No.	Interval between priming and culture	No. tubes exposed to 5 mg/ml BSA 0–6 days	No. of tubes* showing:		
			No response	Burst	Sustained low response
	months				
88	2	6	2	2	2
1-33	2.6	3	2	1	0
2-17‡	3	9	7	1	1
2-50‡	3	9	3	0	6
1-15	3.5	2	2	0	0
1-91	4	9	0	0	9
26	4.5	11	11	0	0
99	4.5	6	3	1	2
30	5	9	9	0	0
2-15‡	6	10	8	0	2
1-85‡	7	5	2	2	1
1-14	10	3	3	0	0

* 5 mg/ml BSA, 0–6 days exposure.

‡ Pooled nodes from two animals.

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tubes, showing that this high serum albumin concentration had no toxic effect.

Heterogeneity among Inhibited Tubes—Although each stimulated tube fluctuated in titer from one medium change to another, the antibody production

TABLE V

Heterogeneity in the Response of Primed Nodes to Various Regimens of Antigen

Rabbit No.	Treatment	Tube	Hemagglutination titers on day of culture indicated											Scoring
			6	7	8	9	10	11	12	14	17	22	24	
2-17-2-18	5 mg/ml BSA 0–6 days	1		0			0		*	0	2	0	0	NR
		2		0			0		0	1	2	1	0	NR
		3		0			0		0	0	1	0	0	NR
		4		0			0		0	1	2	0	0	NR
		5		0			0		0	1	0	0	0	NR
		6		*			0		3	0	0	0	0	B
		7		*			3		5	0	3	2	0	SR
		8		0			0		0	0	0	0	0	NR
		9		0			0		0	0	0	0	0	NR
	0.5 mg/ml BSA 0–6 days	1		0			4		3	0				
		2		0			4		3	0				
		3		0			3		3	3	3	2	0	
		4		0			4		3	*				
		5		0			4		3	2	1			
1-85	0.5 mg/ml BSA 0–2 hr	1	0	0	2	4	5	5	5		5			
	(stimulated)	2	4	4	5	5	5	6	6		6			
	5 mg/ml BSA 0–6 days	1			0	0	4	3	*		0			SR
		2			0	0	0	0	0		0			NR
		3			0	3	3	5	5		2			SR
		4			0	0	4	0	0		0			B
		5			0	0	0	0	0		0			NR

* Not done; NR, no response; B, burst; SR, sustained response.

was sustained throughout the response, and the titers were fairly consistent between tubes. In contrast, the 5 mg/ml BSA 0–6 day tubes showed no such consistency. In five experiments, all of the tubes given the inhibitory treatment (41 tubes) yielded negative titers throughout the duration of the experiment. In eight experiments, a portion gave uniformly negative titers (27 of 57 tubes), but others (23 tubes) showed sustained low titers. A minor portion (7 tubes)

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gave "bursts" of antibody production; that is, the appearance of an abrupt titer in one medium change on or near the peak titer day of the controls, followed by negative titers for the duration of the experiment. A summary of these experiments is shown in Table IV, and Table V enumerates the titers shown by each tube in two different experiments, to show the method of scoring the tube response.

Delay of the Response.—If the 5 mg/ml BSA medium was left in the tubes from day 0–3 and then washed-out, a response occurred in several cases with titers as high as the positive controls (Table VI). This response, however, reached its peak 1–3 days later than the response given by the stimulated controls. More careful study is necessary to ascertain whether the onset of the response is also delayed.

TABLE VI

Delay in Day of Peak Titer Related to Decreased Duration of Exposure to 5 mg/ml BSA

Rabbit No.	Duration of exposure to 5 mg/ml BSA	Day of response peak		Titer* on peak day	
		5 mg/ml	Stimulated	5 mg/ml	Stimulated
	days				
99	0–4	13	9	7	5
	4‡–6	13		6.3	
1-15	3‡–6	12	10	3	3.5
	0–3	13		3	
1-91	0–3	20	10	5	7
	3‡–6	10		3.5	
2-34	0–3	13	8	4	5.4

* Passive hemagglutination titers, expressed as reciprocals of the $\log_2$ dilution.

‡ First exposure to antigen; no initial stimulus on day 0.

DISCUSSION

This paper describes experiments which show that a state of unresponsiveness to BSA can be induced in vitro in primed rabbit lymph nodes by exposing the fragments to 5 mg/ml BSA for the first 6 days of culture. In this system, a response is not detectable until about 6 days after stimulation; therefore the inhibitory antigen may be removed from the tubes on the 6th day after culture, and should not interfere with the subsequent assay of antibody production.

To verify this last assumption, several reconstruction experiments were performed in which 5 mg/ml BSA was added to the stimulated tubes at various times prior to wash-out on the 6th culture day. This amount of BSA, when added as early as 1 day after culture and left in contact with the nodes for 5

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days, failed to interfere with the assay of the subsequent response by hemagglutination or antigen-binding assays.

The injection of high levels of antigen into primed animals may bring about unresponsiveness in two ways; by paralyzing the primed cells, or by exhausting them. In the former case, all antibody production would be inhibited; in the latter, there would be an initial phase of antibody production by the primed cells, followed by a temporary unresponsive state until more Y cells could be recruited. A previous report described experiments on exhaustion, demonstrating that this characteristic sequence of events can occur following injection of sufficient antigen into a primed animal (16).

However, in the experiments reported here, the concentration and duration of antigen exposure was sufficient to result in paralysis. No antibody production was revealed in the inhibited tubes, either after wash-out or during the initial 6 day exposure to the high antigen dose, as measured by the modified Farr assay. Therefore, exhaustion may be excluded as the cause of the unresponsiveness.

The inhibition was characterized with respect to dose, duration, specificity, and completeness. The dose of BSA necessary for the inhibition is greater than 1 mg/ml, and the exposure period has to be longer than the first 3 days of culture, to achieve complete inhibition. The high BSA concentration did not interfere with the production of antibody to other antigens.

Most other investigators reporting secondary paralysis have shown that, as the stimulatory antigen concentration is increased, the magnitude of the resulting response is first increased, then reaches a plateau; still higher antigen concentrations reduce the response to a fraction of the normal. This might be caused by a partial inhibition of each active cell, or alternatively by an all-or-none response of a heterogenous population of cells, some of which are more resistant to inhibition. The latter notion is supported by our findings that within one experiment, a small proportion of the "inhibited" tubes give a sustained low, or normal response, and occasionally a tube displays a 1 day "burst" of antibody synthesis. Apparently, there are some primed cells which cannot be inhibited by this regimen.

It is not known whether a paralyzed cell is actually killed, or only held in a static state, which can be reversed when the antigen concentration is reduced sufficiently to allow induction. A delay in the progression of characteristic early biochemical and cellular events of the immune response, as well as in actual antibody production would be the expected empirical findings associated with reversibility of paralysis. The evidence is that certain of these features are inhibited and/or delayed by the exposure of primed cells to high antigen levels. Mäkelä and Mitchison (8) and Makinodan et al. (10) noted an early inhibition of cellular proliferation; Mäkelä and Mitchison also found a delay in the peak of antibody production. Iványi et al. (14) demonstrated

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that an actinomycin D–sensitive step in antibody synthesis was delayed by excess antigen. We have shown here that following exposure of primed rabbit lymph node fragments to a high concentration of BSA for only the first 3 days of culture (instead of the first 6), the subsequent peak of the response is delayed 1–3 days. These results support the idea of reversible inhibition. In light of this conclusion, the fact that the node fragments which were exposed to antigen for 6 days were subsequently unable to respond, may be explained by our inability to induce responses in cultured primed fragments later than the 4th day after preparation.

What is the relationship between stimulation and paralysis? Either antigen first "orders" the memory (Y) cell to begin the response but at some subsequent step a high level of antigen is able to countermand the order or, alternatively, the cell may be either stimulated or paralyzed, depending on the level and state of antigen that reaches it first, but once stimulated it can no longer be inhibited by antigen. To determine which of these alternatives was true, we first stimulated fragments, waited various intervals, and then added 5 mg/ml BSA until day 6 of culture. If the first explanation were correct, at some time after stimulation and before antibody appearance, the BSA addition should result in a state of paralysis; if the second were correct, even the addition of this BSA soon after the initial 2 hr stimulation would not cause paralysis.

It was found that the immediate addition of 5 mg/ml BSA after stimulation inhibited less than ½ of the tubes, and if the addition was delayed until 1 day after stimulation, none of the tubes were inhibited. It thus appears probable that stimulation is irreversible, although the length of time necessary for optimal stimulation to occur must vary between 2 hr and 1 day for different fragments.

Cellular and molecular explanations of secondary (Y cell) paralysis are few. The existence of an antibody-like receptor bound to the surface of the Y cell is probable (25, 26), but the nature of the initial interaction between antigen and the receptor, which presumably triggers the response, is difficult to fathom. Ultimately, inhibition of the trigger step may be explicable in terms of antigen-receptor combinations being unproductive in antigen excess; whether this is due to an allosteric effect upon the receptor, or to the physical-chemical nature of the aggregate produced upon antigen-receptor combination, awaits further study.

SUMMARY

A concentration of 5 mg/ml bovine serum albumin (BSA) prevents the in vitro elicitation of a secondary response in primed rabbit popliteal lymph nodes, if it is left in contact with the node fragments for the first 6 days of culture. No antibody formation can be detected at any time during the culture

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period in most cases, although occasional fragments are resistant to inhibition. Reducing the exposure time to the first 3 days of culture delays the peak of the antibody response. The inhibition is antigen specific.

Reconstruction experiments demonstrate that the inhibition is not due to antigen masking of the antibody. Even shortly after optimal stimulation, the addition of 5 mg/ml BSA to the fragments was not able to prevent a normal antibody response.

The implications of these findings are that (*a*) a high antigen concentration suspends the memory cell in a reversibly paralyzed state, (*b*) memory cells have a heterogeneous susceptibility to inhibition, (*c*) once induced, the antibody response cannot be inhibited by antigen overloading, (*d*) unresponsiveness in a primed animal can be due to either exhaustion of the memory cell population or paralysis of the memory cell.

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