

HIGH-DOSE DELAY OF THE IMMUNE RESPONSE  
EFFECT OF ACTINOMYCIN D ON CONTINUATION OF THE IMMUNE  
RESPONSE IN VITRO

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It has been demonstrated previously (1, 2) that the appearance of antibody-producing cells is delayed for several days if a high dose of human serum albumin is used as an antigenic stimulus for the chicken. If the high concentration of antigen is maintained, a state of immunological tolerance is established (2, 3). This model seemed to present an opportunity to study in detail the events which occur during induction of a high dose immunological tolerance; particularly, to determine at which step the processes in the immunocompetent cell are altered by antigen so that unresponsiveness is induced rather than immunity. In view of the evidence that neither in long-term tolerance (4) nor during the "delay period" (1) is specific antibody apparent intracellularly, it was necessary to turn to earlier antigen-induced processes of lymphoid cells, e.g., to those which are concerned with their initial biochemical maturation.

In this study an attempt was made to gain information about the effect of antigen dose on the kinetics of specific RNA formation, which is an obligatory step in immunoglobulin synthesis (5). Actinomycin D was used because of its known interference with DNA-dependent RNA synthesis in mammalian cells (6). The importance of actinomycin-inhibitable RNA for antibody synthesis in chicken spleen was determined by serological measurements of its protein product. This functional approach seemed to be the most direct for obtaining information about RNA involved in specific antibody synthesis. It was particularly necessary in our experiments to exclude the possible interference of nonspecific synthesis, which might occur even while antibody production was suppressed (7).

The use of tissue culture for continuation of the response by spleen fragments, from chickens injected with various doses of protein antigen, served to place the lymphoid tissue in an environment free from extracellular antigen and provided defined conditions for treatment with drugs. Moreover, these conditions seemed to give assurance that the results would not be affected by (a) newly induced competent cells, as induction of the primary response is not

possible under these conditions; (b) regulatory feedback mechanisms which are absent in this *in vitro* system.<sup>1</sup> Successful experiments on the immune response in cultured chicken spleen fragments have been published earlier by Patterson et al. (8).

### *Materials and Methods*

White Leghorn chickens, 4-6 months old, or adults, 1-2 yr old, were used for immunization. Single intravenous injections of various doses of human serum albumin (HSA) (Pentex, Kankakee, Ill.) were weight adjusted and expressed per kg body weight. At different times after primary or secondary immunization, chickens were sacrificed by decapitation and spleens were removed aseptically. The time between sacrifice and initiation of tissue culture varied between 30 and 90 min.

Spleens deprived of their membrane were cut into fragments with scissors for 30-60 sec, in a beaker. The fragments were resuspended in 10-20 ml of Hanks' solution with 5% normal chicken serum (NCS) and poured into a Petri dish. The supernatant with free cells was removed by suction, and the remaining fragments were washed several times with Hanks' solution. There was no significant difference in immune response when the fragments were washed three or six times. 10-13 fragments of 1 mm<sup>3</sup> were transferred to a plastic organ culture dish (Falcon Plastics, Los Angeles, Calif.) with a circumferential wet chamber and a central basin of 2 cm in diameter. The basin was filled with 0.5 ml of Minimum Essential Eagle's medium containing 1  $\mu$ M hydrocortisone, 0.1 mM penicillin, 0.04 mM streptomycin and 20% NCS.

Fragments were placed on the surface of a wire grid triangle in the basin, so that they were partially covered by medium having the upper part free for aeration. The cultivation took place at 37°C in a 5% CO<sub>2</sub>, 95% air atmosphere.

Preliminary experiments have shown that the use of larger fragments, or a greater number, did not increase but rather decreased the titer of antibodies obtained *in vitro*. Egg white or ovalbumin, if substituted for NCS as the protein component of the medium, caused a lower titer of antibodies *in vitro*; we could not confirm the reported enhancing effect of egg white protein on the immune response in tissue culture (9). If medium without protein was used, the response was decreased approximately fourfold.

*Drug Treatments.*—Actinomycin D (AMD) was generously supplied by Merck, Sharpe & Dohme, Rahway, N. J. Before the onset of tissue culture, AMD was added at a 1 or 5  $\mu$ g/ml concentration for 60 min at room temperature, followed by two washings with Hanks' solution. In other experiments, AMD was added to the tissue culture medium at a concentration of 1  $\mu$ g/ml continually during the whole cultivation period.

At a level of 1  $\mu$ g AMD/ml, the incorporation of <sup>3</sup>H-leucine into protein and <sup>14</sup>C-uridine into RNA was reduced to one quarter of the control values after 1 day of culture, using a filter paper disk technique for the incorporation studies (10). Further work on the effect of AMD on chicken spleen macromolecular synthesis will be published elsewhere.

Cycloheximide (Calbiochem, Los Angeles, Calif.) was added to the tissue culture medium at a concentration of 10  $\mu$ g/ml.

*Antibody Assays.*—Passive hemagglutination with HSA-coated, sheep red cells was performed in microhemagglutination plates, as described previously (11). Tannic acid or bis-

<sup>1</sup> Iványi, J., C. Lippincott, L. Holtz, and E. E. Sercarz. The type of antibodies involved in feedback inhibition of the immune response to low doses of HSA in the chicken. Submitted for publication.

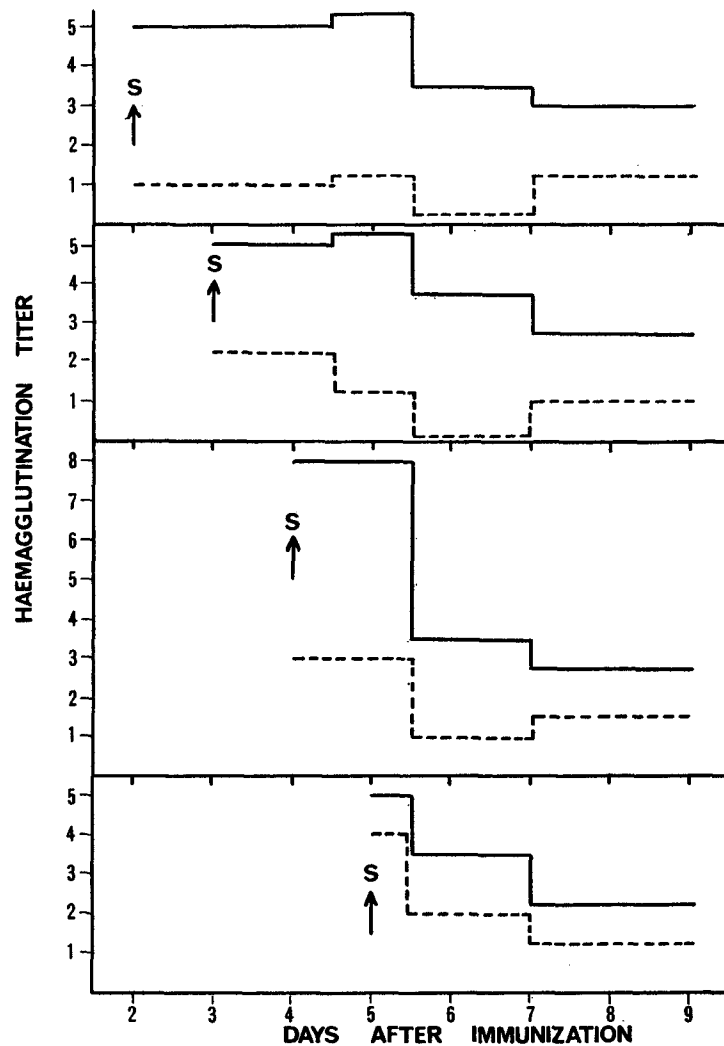


FIG. 1. Effect of AMD on the continuation of the primary response *in vitro*. Splenectomy on different days after immunization with 20 mg HSA is indicated by the arrow (S), which marks the onset of tissue culture. HA titers represent mean values for 10 parallel untreated cultures (—) and four cultures treated with 5  $\mu\text{g}/\text{ml}$  AMD for 60 min before the onset of tissue culture (---).

diazotized benzidine was used for attachment of the HSA. Titers are expressed as the log to the base 2, i.e., as the number of wells giving positive agglutination after doubling dilutions of media.

The determination of antigen-binding capacity (ABC) by the Farr test (12) was performed with undiluted media: 0.4 ml media was incubated with 0.2 ml  $^{125}\text{I}$ -HSA containing 0.05  $\mu\text{g}$

$^{125}\text{I}$ -HSA (with a specific activity of approximately  $5 \times 10^5$ - $10^6$  cpm/ $\mu\text{g}$ ) at  $37^\circ\text{C}$  for 60 min and  $0^\circ\text{C}$  overnight. 0.5 ml saturated ammonium sulfate solution was added at  $0^\circ\text{C}$ , centrifuged in the cold for 30 min, and washed with 2 ml of 45% ammonium sulfate, centrifuged, and resuspended in 0.5 ml water for counting. The radioactivity of precipitates was measured in a Baird-Atomic crystal well scintillation counter and the results expressed as the percentage of the added  $^{125}\text{I}$ -HSA present in the washed precipitate.  $^{125}\text{I}$ -HSA was labeled by a modification of the chloramine-T method (13).

TABLE I  
*Absence of In Vitro Antibody Response after Injection with a High Dose of HSA*

Dose of HSA injected i.v.	Splenectomy days after injection	Treatment of spleen fragments	Antibody response in vitro			
			HA*		ABC†	
			—	ME	—	ME
20 mg	2	none	5.3	0	51.9	13.3
		AMD	1.2	0	7.9	9.0
	3	none	5.3	0	67.2	33.9
		AMD	1.2	0	12.8	9.4
	4	none	8.0	0	91.8	49.1
		AMD	3.0	0	35.4	—
	5	none	5.0	0	76.5	40.9
		AMD	4.0	0	86.4	49.7
2000 (day 0)	2	none	0	0	6.4	
	3		0	0	6.0	
1000 (day 1)	4		0	0	6.7	
	5		0	0	6.9	

\* HA titers are mean values at the peak antibody response in vitro from 10 parallel cultures (without treatment) or 4 cultures (fragments treated 1 hr with 5  $\mu\text{g}$  AMD/ml).

† ABC values were determined with pooled media.

Treatment of media with 0.1 M 2-mercaptoethanol (ME) was performed for 60 min at  $37^\circ\text{C}$ . This was shown to distinguish between the major components of 7S and 19S molecular types of antibodies in this system (14, 11). Representative samples were checked by ultracentrifugation on sucrose gradients, under conditions described previously (11).

## RESULTS

*Effect of AMD on Spleen Fragments Removed at Different Times after Immunization.*—Four chickens, 4 months old, were injected with 20 mg HSA and sacrificed on the 2nd, 3rd, 4th, or 5th day after immunization. Washed spleen fragments were untreated or treated with 5  $\mu\text{g}/\text{ml}$  AMD in Hanks' solution

for 1 hr. Tissue culture media were changed on successive days and hemagglutination (HA) titers are given in Fig. 1. The peak HA titer of untreated cultures was found on the 5th day after immunization followed by a gradual decrease. There was a clear-cut difference in the titers of the first media removed from AMD-treated fragments. The early (2nd day) spleen response was almost completely inhibited; in spleens removed on the 3rd and 4th days,

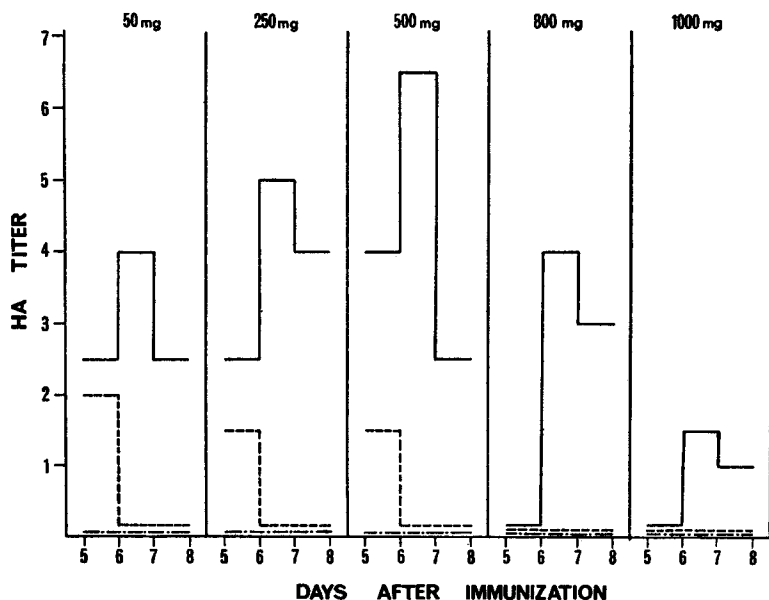


FIG. 2. Effect of AMD and cycloheximide on the primary response induced with various doses of antigen. The results represent one chicken for the doses of 250, 500, and 1000 mg, two chickens for 800 mg, and three chickens for 50 mg; mean values are presented throughout. All chickens were sacrificed on the 5th day after immunization. Mean HA titers of four parallel cultures: untreated (—), in the presence of 1  $\mu$ g AMD/ml medium (---), or 10  $\mu$ g cycloheximide/ml medium (-·-·-).

there was a partial inhibition and on the 5th day spleen culture was almost identical with the untreated control. A similar shift from AMD sensitivity to AMD resistance was apparent after the next change of media.

The AMD-sensitive to AMD-resistant "shift" was confirmed when the media were tested for antigen-binding capacity (Table I). Comparative data using both methods, with or without ME-treatment, demonstrated that ME-resistant, presumably 7S, antibodies were detected only with the Farr technique. All HA antibodies were ME-sensitive, with 19S sedimentation characteristics by sucrose gradient centrifugation.

There was a slight but evident recovery from inhibition in AMD-treated cultures of the 2nd–4th day spleens on the 7th day after immunization.

In the same experiment, four chickens were injected with a high dose of HSA: 2000 mg on day 0 and 1000 mg on day 1. Spleens removed on the 2nd–5th days were cultured until the 9th day. Their response, determined by the HA and Farr assays, is presented together with peak values of the 20 mg-injected group in Table I. The data show a complete lack of response *in vitro* after immunization with the massive dose of HSA.

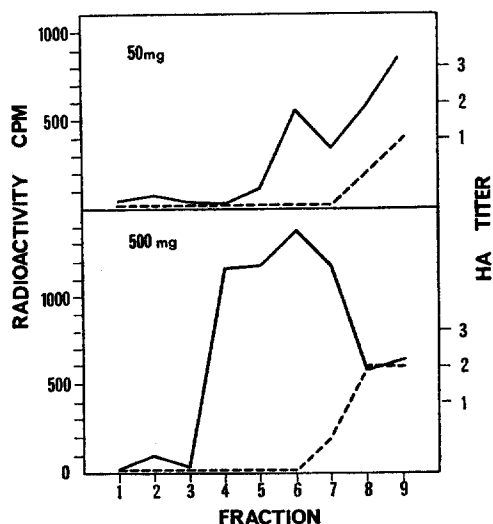


FIG. 3. Molecular type of antibodies formed *in vitro*. Sucrose gradient fractions of media at the peak of the *in vitro* response, from the experiment presented in Fig. 2: top, 50 mg HSA; bottom, 500 mg HSA; (—), Farr test; (---), HA titer.

*Effect of AMD on Spleen Fragments Removed at One Interval after Various Doses of HSA.*—Eight chickens, 4 months of age, were injected with 50, 250, 500, 800, or 1000 mg HSA. For sacrifice the 5th day was chosen, because in the previous experiment the immune response to 20 mg HSA was AMD-resistant by that time. Cultivation *in vitro* was performed in the presence of 1  $\mu\text{g}/\text{ml}$  AMD or 10  $\mu\text{g}/\text{ml}$  cycloheximide, and in control cultures. The results presented in Fig. 2 demonstrate that, in untreated cultures, the peak response occurred after immunization with 500 mg HSA. However, the proportion of the response resistant to AMD after that dose was smaller than in cultures from chickens injected with 50 mg HSA. Thus, after injection of 500 mg HSA, the AMD-sensitive period persists longer; expressed alternatively, the onset of the AMD-resistant response is delayed. In cultures from chickens injected

with 800–1000 mg HSA, no antibodies were found at the first change of medium (6th day). On the next day antibodies appeared, but only in untreated cultures. Media with cycloheximide were negative in all instances, indicating that antibody, when found, reflected true *in vitro* synthesis.

It was surprising to find that the HA antibody, which is formed *in vitro* after high doses of antigen, was ME-sensitive in the primary response, because at that same dose of HSA, high titers of ME-resistant HA antibodies are formed *in vivo* (11). However, we were able to demonstrate 7S antibodies by the Farr technique, which apparently would not show positive hemagglutination (see Fig. 3).

TABLE II  
*The Effect of Actinomycin D on Recovery of the In Vitro Response Induced by 500 mg HSA*

Dose of HSA injected i.v.		Treatment during cultivation	HA titer in media	
Day 0	Day 2		3–4 day	4–6 day
<i>mg</i> 0.5	<i>mg</i> 0	none AMD	2 0	1.5 0
500.0	0	none AMD	0 0	3 0
0.5	500.0	none AMD	0 0	3.5 0

Spleens removed on 3rd day after immunization were cultured in quadruplicate, duplicates with or without 1  $\mu$ g AMD/ml medium.

Another experiment was performed to confirm that the delayed *in vitro* response in cultures from chickens injected with a high dose of HSA was still AMD-sensitive at its onset. Three chickens 4 months old were injected with 0.5 mg HSA, 500 mg HSA, or 0.5 mg HSA on day 0 and 500 mg on day 1, respectively (Table II). After splenectomy on the 3rd day, culturing was performed with or without the presence of 1  $\mu$ g/ml AMD. Cultures of both chickens injected with 500 mg HSA had no HA titers on the 4th day and during the next 2 days (4th–6th days) antibodies were formed only in cultures not treated with AMD.

*Continuation of the Anamnestic Response In Vitro: the Effect of AMD.*—The spleen of an adult hen, preimmunized twice and challenged with 50 mg HSA, was removed on the 2nd day after immunization. Fragments were cultured in media containing 20% NCS or without protein. The response *in vitro* was biphasic, with peaks on the 4th and 7th days after immunization. The first peak was ME-sensitive; the second was ME-resistant, in the characteristic

7S position after ultracentrifugation in a sucrose gradient. The normal chicken serum in the medium resulted in HA titers two tubes higher than the protein-deprived cultures.

Four 6 month old chickens, preimmunized 3.5 months earlier with 50 mg HSA, were challenged with 20–2000 mg HSA. Spleens were removed on day 1 or 4.5 after challenge. The spleen cultures initiated on the 1st day after challenge did not have detectable HA antibodies during the 4 day period of cultivation in vitro (Table III). However, significantly positive antigen binding

TABLE III  
*Effect of Antigen Dose and Time of Splenectomy on the Continuation of the Secondary Response In Vitro*

Dose of HSA injected i.v.	Splenectomy days after injection	Treatment of spleen fragments	Antibody response in vitro		
			HA		ABC
			—	ME	
<i>mg</i>					%
20	1	none	0	0	46.0
		AMD	0	0	4.1
	4.5	none	5	3	100
		AMD	4	2	100
2000	1	none	0	0	5.7
		AMD	0	0	3.5
	4.5	none	0	0	6.2
		AMD	0	0	6.4

Mean values from 10 cultures, 4 treated and 6 untreated with 1  $\mu$ g AMD/ml for 1 hr. Cultures were maintained in vitro 4 days.

was detected in media of the 20 mg HSA spleen cultures. In the spleen cultures started 4.5 days after a 20 mg challenge, 100% ABC values were obtained (dilution of media was not performed). The effect of AMD on the “early” and “late” spleen was similar to that found in the primary response. However, no response could be detected during the 4 day in vitro cultivation of spleens from chickens challenged with 2000 mg HSA.

Another two 4 month old chickens, preimmunized 6 wk earlier with 500 mg HSA, were challenged with 0.5 or 500 mg HSA and sacrificed on the 2nd day after challenge. Tissue cultures were set up either untreated or with 1  $\mu$ g/ml AMD given at the beginning of cultivation (2nd day) or withheld until 2 days later (4th day). Media were tested with both the HA and Farr methods and the results are given in Fig. 4. The responses stimulated with the 0.5 and 500



mg HSA doses showed different sensitivities to AMD treatment: the response (HA and ABC) of the "low dose" spleen was already to a large extent AMD-resistant, while the response of the "high dose" spleen was AMD-sensitive at that time. The formation of HA antibodies seemed to be more inhibited than antibodies with antigen-binding potency. The dependence of the AMD effect on the challenge dose of antigen was similar to that found in the primary response, representing a delayed shift from AMD sensitivity to resistance after the higher dose of antigen used for stimulation.

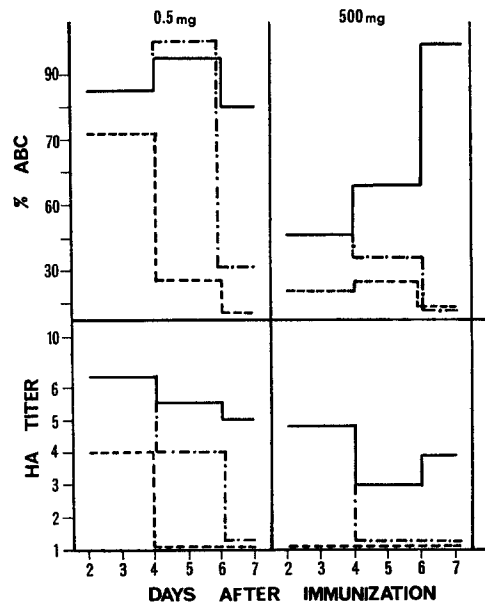


FIG. 4. Continuation of the secondary response in vitro after different doses of antigen for challenge. Tissue cultures were untreated (—), or treated with  $1 \mu\text{g}$  AMD/ml medium added at the start of tissue culture (---) or 2 days later (— · —) or 2 days later (· · · ·). HA titers represent mean values of 4 or 6 parallel cultures; Farr tests were performed with pooled media.

The cultures in which AMD was withheld for 2 days clearly demonstrate that the low dose response achieved AMD-resistance earlier than the high dose response. It would require withholding AMD for 4 days to see whether the high dose response becomes truly AMD-resistant during in vitro culture.

The decreasing titer of HA antibodies and the increase of ABC values during the 5 days of cultivation in vitro (Fig. 4) indicates that the biphasic response demonstrated in an earlier experiment is also expressed here by the initial production of HA antibodies, followed later by antigen-binding, Farr test-positive antibodies.

## DISCUSSION

We have shown that the delay in the appearance of antibody after a high dose of HSA reflects a simultaneous delay in at least one of the early steps necessary in the immune response, a step which is inhibitable by actinomycin D. We have demonstrated in our experiments that a change in the sensitivity to AMD treatment occurs during the immune response. In spleen fragments removed early after immunization, AMD completely suppressed anti-HSA formation, while in spleens removed later, the immune response was largely resistant to AMD.

The *in vitro* continuation of the immune response has previously been shown to be inhibited by low concentrations of AMD (0.5–1.0  $\mu\text{g}/\text{ml}$ ) (5, 15, 16). Wust and Hanna (17) have reported the interference by AMD with the early inductive period of the immune response. Ambrose has shown that, whereas 0.01  $\mu\text{g}/\text{ml}$  AMD can prevent the *in vitro* elicitation of the secondary response, its addition to the culture medium later, during the productive phase of the response, inhibits antibody formation only gradually over the course of several days (18). This inhibition occurs *after* the major cell division preparatory to the secondary response has occurred in this rabbit lymph node system (19).

Thus the evidence implicates a relatively short-lived RNA whose presence is required for continued antibody synthesis. Studies on myeloma tumors with labeled RNA precursors, observing the sucrose gradient characteristics of the synthesized material, suggest that immunoglobulins are synthesized on polyribosomes, presumably held together by strands of RNA (20, 21).

It is clear from the radioautographic studies of Mitchell (22, 23) that the intensive RNA synthesis of blast cells contrasts with the negligible incorporation into lymphocytes and plasma cells. This had been demonstrated previously *in vivo* (24) where, despite a low uptake of labeled RNA precursors, plasma cells showed active protein synthesis without concomitant RNA synthesis. The general picture derived from these results is that RNA is sufficiently stable *not* to have to be continually synthesized during the major phase of antibody production in plasma cells. During immunocyte maturation, there is a morphological shift from immature, RNA-producing blasts to mature, protein-producing plasma cells (2, 22). Thus the RNA synthesis during the early blast conversions would be actinomycin D-sensitive, while protein synthesis with *in vivo* preformed RNA would be relatively actinomycin D-resistant. The presence of preformed RNA templates, allowing unimpaired or even increased antibody protein synthesis, can be inferred from other studies during which AMD markedly inhibited new RNA synthesis. Bussard (25) found the induction of an *in vitro* primary response was completely suppressed in cells pretreated with AMD, while the continuation of the response of lymph node cells from preimmunized rabbits was inhibited only partially during the first 20 hr of incubation *in vitro*.

Varying reports have been published in attempting to estimate the half-life of an RNA specific for a particular antibody (5, 15, 26). The estimates obtained range from less than 12 hr (15) to as long as 10 days (26). The probable heterogeneity of template RNA stabilities has been shown in several reports. Mitchell (23) has demon-

stated that the inhibition of RNA synthesis in blast cells increased from 30 to 75% when the concentration of AMD was changed from 0.5 to 2  $\mu\text{g/ml}$ , suggesting that different kinds of RNA are synthesized in blast cells. Stavitsky and Gusdon's studies also point to at least two classes of mRNA's with different lifetimes (16). In nonantibody systems, template RNA with differing stabilities has been demonstrated in the biosynthesis of hormones (27, 28). Moreover, the existence of another type of RNA is suggested in immature differentiating cells, during embryogenesis (29, 30). This "differentiation-controlling RNA" was found to be highly susceptible to AMD and unstable, in contrast to the AMD-resistant, stable RNA functioning as template in the protein synthesis of differentiated cell types. Since differentiation and maturation of lymphoid cells are basic processes in the immune response, and both RNA and protein synthesis occur in cells at different stages of maturation, further information on the spectrum of RNA types and their functions seems to be required before the control of antibody systems can be understood at this level.

However, we have demonstrated that with increasing doses of HSA, the AMD-sensitive period of response *in vitro* was delayed or extended (Figs. 2, 4). We may assume that the *in vitro* sensitivity to AMD represents a shortage of preformed mRNA, suggesting that the onset of mRNA synthesis is delayed with high doses of antigen. After immunization with doses of 500–1000 mg HSA, there was a delayed onset of detectable antibody production *in vitro*. During 24–48 hr of cultivation *in vitro*, there was a recovery of antibody formation which was absent in AMD-treated cultures. This indicates that synthesis of some component, presumably mRNA, is necessary for the recovery of immunity.

Despite the possibility that AMD sensitivity might reflect the inhibition of some other synthetic entities (e.g., 31, 32), it did not have a nonspecific toxic effect in our culture system, as has been found elsewhere (33). Thus, if the time and dose were appropriately chosen, vigorous antibody formation could be shown in our experiments, even in the presence of AMD. We would like to emphasize this point: that whatever the molecular nature of the AMD target, the high dose of HSA seemed to delay simultaneously any sign of an immune response *in vivo* (2) and also delayed the AMD-sensitive step in antibody formation. Other evidence of the controlling role of antigen dosage for RNA formation has been reported by Pagoulatos (34) who found that protein synthesis by rabbit ribosomes was inhibited on the 3rd day after challenge with ovalbumin if on the 2nd day another 50 mg of ovalbumin was injected.

It is tempting to consider that the delay of the immune response in our system implies the existence of temporarily suppressed "tolerant cells," whose macromolecular synthesis necessary for anti-HSA formation has also been delayed. However, although spleen fragments continued their response *in vitro* between the 2nd and 5th day after immunization with a medium dose of antigen, if a high dose of HSA (3000 mg) was administered, the subsequent immune response *in vitro* was absent. Other authors have shown that lymphoid cells from animals after or during induction of immunological tolerance do not produce antibodies when transferred to immunologically inert recipients (35, 36, 37) or in tissue culture (38, 39). On the other hand, French workers have

reported success recently in finding a recovery of immunity when "tolerant cells" were cultured *in vitro* (40).<sup>2</sup> The absence of immunological recovery in our system, when spleen tissue was removed from the excess of antigen present *in vivo*, may indicate a similarity of mechanisms responsible for induction of the short term "delay" of the immune response, and long lasting immunological tolerance. Despite extensive washing of fragments (six times) before initiation of the tissue culture, it is still possible that an excess of intracellular antigen, perhaps in macrophages, might be transferred into tissue culture and block the synthesis or release of antibodies. Firm conclusions about the existence of antigen-suppressed or tolerant cells with a potential for recovery, would require ideal conditions of *in vitro* cultivation for extended periods of time, and our limitations in this respect are evident. However, the *in vitro* recovery of responsiveness at lower dose levels (e.g., in the 800 mg chickens [Fig. 2] on the 6th day after immunization and the 2nd day of culture) must have taken place in cells initially inhibited and not by cells recruited from some extrasplenic source. Since the recovery of responsiveness in that experiment did not take place in the presence of cycloheximide (which prevents new protein formation by preventing the transfer of amino acids from aminoacyl tRNA), this provided evidence that recovery was not simply due to the release of preformed antibody.

Whereas *in vivo*, 500 mg HSA stimulates a maximal IgG response and a markedly inhibited IgM response, in our *in vitro* experiments a relatively high IgM and poor IgG primary response was observed. If IgG antibodies have a feedback inhibitory role in shutting off IgM synthesis, the lack of appropriate culture conditions for IgG synthesis might then rationalize our continued IgM synthesis. Our previous findings<sup>1</sup> about the lack of antibody feedback *in vitro* are in agreement with this explanation.

In the secondary response, mainly IgG antibodies were formed *in vitro* and therefore the Farr test turned out to be the most sensitive antibody assay (Table III). The success in demonstrating the sequential appearance of two molecular types of antibodies in this system, in contradistinction to rabbit lymph node culture, makes this chicken model especially suitable for further study of immunocyte maturation.

#### SUMMARY

The continuation of the primary and secondary antibody response to human serum albumin (HSA), induced *in vivo*, was followed in explanted chicken spleen fragments. The effect of actinomycin D (AMD) on the *in vitro* response was studied in spleens from chickens injected with various doses of HSA and removed at differing intervals after injection.

The antibody response of "early spleen" cultures was AMD-sensitive, while

<sup>2</sup> Paraf, A., and A. Bourgois. Information Exchange Group Memo No. 208.

cultures of spleens removed later were AMD-resistant. It was suggested that this shift represented the development of cells with in vivo preformed RNA involved in specific immunoglobulin synthesis.

With increasing doses of HSA, the AMD-sensitive phase was prolonged, suggesting the delay of mRNA formation or some other AMD-inhibitable process in vivo. With large doses of HSA, the immune response in vitro was decreased, starting after a 1-2 day delay and not occurring in the presence of AMD. Massive doses of HSA completely inhibited the continuation of the response in vitro by spleen fragments removed between the 2nd and 5th day after injection.

The results point to the controlling role of antigen dose in determining the onset of macromolecular synthesis during immunocyte maturation.

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#### BIBLIOGRAPHY

1. Černý, J., J. Iványi, J. Madár, and T. Hřaba. 1965. The nature of the delay in the immune response after administration of large doses of protein antigen in chicks. *Folia Biol. Prague*. **11**:402.
2. Černý, J., and J. Iványi. 1966. The dose of antigen required for the suppression of the IgM and IgG antibody response in chickens. II. Studies at the cellular level. *Folia Biol. Prague*. **12**:343.
3. Iványi, J., T. Hřaba, and J. Černý. 1964. Immunological tolerance to human serum albumin in chickens of adult and juvenile age. *Folia Biol. Prague*. **10**:198.
4. Sercarz, E., and A. H. Coons. 1959. Specific inhibition of antibody formation during immunological paralysis and unresponsiveness. *Nature*. **184**:1080.
5. Uhr, J. W. 1963. Actinomycin D: its effect on antibody formation in vitro. *Science*. **142**:1476.
6. Reich, E., R. M. Franklin, A. J. Shatkin, and E. L. Tatum. 1961. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. *Science* **134**:556.
7. Hanan, R., and J. Oyama. 1954. Inhibition of antibody formation in mature rabbits by contact with the antigen at an early age. *J. Immunol.* **73**:49.
8. Patterson, R., I. M. Suszko, and J. J. Pruzansky. 1964. In vitro production of chicken globulins and precipitating antibody. *Immunology*. **7**:440.
9. Patterson, R., I. M. Suszko, and J. J. Pruzansky. 1966. Heterogeneity of antibody-producing cells and antibody as a result of environmental alterations in tissue culture. *J. Immunol.* **96**:139.
10. Bollum, F. J. 1966. Filter paper disk techniques for assaying radioactive macromolecules. *In* Procedures in Nucleic Acid Research. G. L. Cantoni and D. R. Davies, editors. Harper and Row, N. Y. 296.
11. Iványi, J., V. Valentova, and J. Černý. 1966. The dose of antigen required for the suppression of the IgM and IgG antibody response in chickens. I. The kinetics and characterization of serum antibodies. *Folia Biol. Prague*. **12**:157.

12. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I\*BSA and antibody. *J. Infect. Diseases.* **103**:239.
13. McConahey, P., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Intl. Arch. Allergy Appl. Immunol.* **29**:185.
14. Benedict, A. A., R. J. Brown, and R. Hersch. 1963. Inactivation of high and low molecular weight chicken antibodies by mercaptoethanol. *Proc. Soc. Exptl. Biol. Med.* **113**:136.
15. Svehag, S. E. 1964. Antibody formation in vitro by separated spleen cells: inhibition by actinomycin or chloramphenicol. *Science.* **146**:659.
16. Stavitsky, A. B., and J. P. Gusdon, Jr. 1966. Role of nucleic acids in the anamnestic antibody response. *Bacteriol. Rev.* **30**:418.
17. Wust, C. J., and M. G. Hanna. 1965. Time relationship of injection of actinomycin D and antigen to the immune response. *Proc. Soc. Exptl. Biol. Med.* **118**:1027.
18. Ambrose, C. T. 1966. Biochemical agents affecting the inductive phase of the secondary antibody response initiated in vitro. *Bacteriol. Rev.* **30**:408.
19. O'Brien, T. F., and A. H. Coons. 1963. Studies on antibody production. VII. The effect of 5-bromodeoxyuridine on the in vitro anamnestic antibody response. *J. Exptl. Med.* **117**:1063.
20. Tawde, S., M. D. Scharff, and J. W. Uhr. 1966. Mechanisms of  $\gamma$ -globulin synthesis. *J. Immunol.* **96**:1.
21. Askonas, B., and A. Williamson. 1966. Biosynthesis of immunoglobulins on polyribosomes and assembly of the IgG molecule. *Proc. Roy. Soc. London, Ser. B.* **166**:232.
22. Mitchell, J. 1964. Autoradiographic studies of nucleic acid and protein metabolism in lymphoid cells. I. Differences amongst members of the plasma cell sequence. *Australian J. Exptl. Biol. Med. Sci.* **42**:347.
23. Mitchell, J. 1964. Autoradiographic studies of nucleic acid and protein metabolism in lymphoid cells. II. The stability and actinomycin sensitivity of rapidly formed RNA and protein. *Australian J. Exptl. Biol. Med. Sci.* **42**:363.
24. Schooley, J. C. 1961. Autoradiographic observations of plasma cell formation. *J. Immunol.* **86**:331.
25. Bussard, A. E. 1966. Antibody formation in nonimmune mouse peritoneal cells after incubation in gum containing antigen. *Science.* **153**:887.
26. Miller, J. J., III. 1964. An autoradiographic study of the stability of plasma cell ribonucleic acid in rats. *J. Immunol.* **93**:250.
27. Seed, R. W., and I. H. Goldberg. 1963. Biosynthesis of thyroglobulin; relationship to RNA template and precursor protein. *Proc. Natl. Acad. Sci. U.S.* **50**:275.
28. Ney, R. L., W. W. Davis, and L. D. Garren. 1966. Heterogeneity of template RNA in adrenal glands. *Science.* **153**:896.
29. Scott, R. B., and E. Bell. 1964. Protein synthesis during development: control through messenger RNA. *Science.* **145**:711.
30. Yaffee, D., and M. Feldman. 1964. The effect of actinomycin D on heart and thigh muscle cells grown in vitro. *Develop. Biol.* **9**:347.

31. Honig, G. R., and M. Rabinovitz. 1964. Actinomycin D inhibition of protein synthesis unrelated to effect on template RNA synthesis. *Science*. **149**:1504.
32. Revel, M., H. H. Hiatt, and J. P. Revel. 1964. Actinomycin D: an effect on rat liver homogenates unrelated to its action on RNA synthesis. *Science*. **146**:1311.
33. Soeiro, R., and H. Amos. 1966. mRNA half-life measured by use of actinomycin D in animal cells—a caution. *Biochim. Biophys. Acta*. **129**:406.
34. Pagoulatos, G. N. 1966. Contribution a l'étude de la biosynthèse des anticorps: immunisation et activation des ribosomes de la rate. *Ann. Inst. Pasteur* **110**:497.
35. Sercarz, E., and A. H. Coons. 1963. The absence of antibody-producing cells during unresponsiveness to BSA in the mouse. *J. Immunol.* **90**:478.
36. Brooke, M. S., and M. Karnovsky. 1961. Immunological paralysis and adoptive immunity. *J. Immunol.* **87**:205.
37. Friedman, H. 1962. Transfer of antibody formation by spleen cells from immunologically unresponsive mice. *J. Immunol.* **89**:257.
38. Friedman, H. 1966. Absence of agglutinin formation in spleen cell cultures from immunologically tolerant mice. *J. Immunol.* **96**:289.
39. Halpern, B. N., R. W. Ferraresi, T. Neveu, and A. Brannelee. 1964. L'absence de synthèse d'anticorps in vitro par les cellules lymphoïdes prélevées chez des animaux in état d'immunoparalysie. *Intl. Arch. Allergy Appl. Immunol.* **25**:145.
40. Hayes, R., and A. Paraf. 1966. Etude de quelques facteurs dans la production d'anticorps en culture cellulaire. *Ann. Inst. Pasteur.* **110**(Suppl.):118.