Actinomycin D before and during Primary and Secondary Anti-Forssman Immunoglobulin Hemolysin Responses in Rabbits*

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Abstract. The effects of actinomycin D were studied on successive stages of the primary and secondary (anamnestic) anti-Forssman immunoglobulin hemolysin responses induced in rabbits by the antigen, sheep red blood cells. (Fully developed anamnestic reactivity was tested 1.5–2 months after the start of an untreated initial response.) The stages of both responses include induction by antigen, the latent period, the acute rise of hemolysin to peak titer, and the decline afterwards. This antibiotic modified both responses significantly in ways strikingly similar to those produced by large doses of x-rays. It was injected in a sublethal amount (0.08 mg/kg) at selected times before or after a test intravenous injection of $10^{9.3}$ sheep erythrocytes/kg. Given within several days before the antigen, it delayed and depressed peak titer, whereas given during induction and the latent period, it delayed and enhanced peak titer. Given during the acute rise, its effect decreased, and given during the decline after peak titer, it only produced erratic slight rises in titer. It is suggested that delayed and depressed peak titers are associated with both known activities of sublethal doses of actinomycin D, i.e., cytotoxicity and inhibition of RNA synthesis, whereas enhanced peak titers are brought about by the presence of nucleic acid degradation products released by the cytotoxicity of actinomycin D at the critical time of active RNA synthesis in the latent period. Effects of actinomycin D differed quantitatively in the two responses. During the initial response, delayed depression was not apparent as soon and did not last as long, whereas delayed enhancement was more pronounced. In terms of the cells operating in the two responses, the data indicate that immunologically competent initial cells, as compared to memory cells, are more easily stimulated, are not injured as quickly, recover more rapidly, and overcompensate for the injury for a longer time. In addition, in untreated controls, recruitment of immunologically competent initial cells appears to be largely inhibited during induction of memory cells. Otherwise, decline of the secondary response would be less abrupt.

Introduction. To attack the problem of antibody formation, hemolysin formation in intact rabbits$^{1,2}$ has previously been analyzed with respect to total body irradiation.$^{3,4}$ Both primary and secondary responses involve primarily the anti-Forssman and not the isophile immunoglobulins when immunization consists of one injection from $10^9.3$ to $10^{9.2}$ sheep red blood cells. They are de-
pressed or enhanced after a delay or stimulated without a delay in a dose-, time-, and response-dependent manner by x-rays. Effects of the analogue, 5-bromodeoxyuridine, have also been examined.\(^6\)

Both responses were treated with actinomycin D in the current work because this antibiotic blocks the synthesis of “messenger” RNA\(^7\) which predominantly takes place during the latent period\(^8\) and has a direct cytotoxic action on lymphoid cells.\(^9\)\(^,\)\(^10\)

In such treated rabbits, slight depression and maximal delayed enhancement of hemolysin formation were encountered in a time- and response-dependent manner, but there was no stimulatory or adjuvant action.

**Materials and Methods.** Experiments usually consisted of 36 young adult female rabbits of the Dutch Belted variety, weighing 2–2.5 kg. Half of these had been injected intravenously 1.5–2 months previously with sheep erythrocytes to allow for the full development of anamnestic reactivity.\(^2\) Small groups of the two sets were tested with actinomycin D at selected intervals before or after an intravenous test injection of 10^8.\(^3\) sheep erythrocytes/kg rabbit. A few untreated controls received sheep red blood cells alone. No rabbit was given more than one dose of actinomycin D or more than one injection of the erythrocytes because of residual effects of early treatment.\(^11\)

Blood (1–2 ml) via the right ear vein was obtained just before the test injection of sheep erythrocytes and 3 or 4 times a week for a month or more thereafter. Serum from each rabbit was stored at \(-20^\circ C\) until they were titrated on the same day to obtain a photometric 50% hemolysis endpoint according to our usual procedure.\(^1\)\(^,\)\(^12\) Titers from each rabbit were plotted semilogarithmically against days after immunization, and lines were fitted to the titers by inspection to obtain characteristic parameters. In fitting the lines in both responses, hemolysin present at the time of injecting the erythrocytes was subtracted to obtain net peak titers and rates \((k)\) as well as relevant times. Declines of hemolysin after peak titer are expressed in terms of half-disappearance time in days \((0.693/k)\). Means for the parameters from similarly treated rabbits were used to construct mean plots in the figures.

Differences between means plus or minus standard errors were considered significant when \(p\) was 0.05 or less. (For fuller details, see recent papers.\(^9\))

Actinomycin D was obtained from Merck, Sharp and Dohme under the trade name of Cosmegen. For injection, this product which contains 0.5 mg of “dactinomycin” and 20 mg of the inactive ingredient, mannitol, to facilitate solubility, was diluted in order with 10 ml of distilled water and 40 ml 0.7% NaCl solution. Such dilutions were used within a week. Meanwhile, they were kept at 4°C. A total dose of 0.08 mg/kg rabbit was divided into four equal increments. Two of these were given intravenously into the left ear and intraperitoneally during the morning hours, and the other two were

**Table 1.** Control hemolysin responses after the intravenous injection of 10^8.\(^3\) sheep erythrocytes/kg rabbit and times of treatment with actinomycin D.

<table>
<thead>
<tr>
<th>Series</th>
<th>Latent period (days)*</th>
<th>Acute Rise Average rate ((k)) to peak</th>
<th>Length in days</th>
<th>Peak titer (log units)</th>
<th>Decline in days ((\mu/s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Mean ± standard errors for 5 parameters in control series P and S</td>
<td>2.8 ± 0.07 1.3 ± 0.12 5.6 ± 0.3 3.46 ± 0.04 8.6 ± 0.6</td>
<td>1.8 ± 0.03 2.1 ± 0.12 3.2 ± 0.2 3.59 ± 0.04 6.1 ± 0.4</td>
<td></td>
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<tr>
<td>Primary (P)</td>
<td>2.8 ± 0.07 1.3 ± 0.12 5.6 ± 0.3 3.46 ± 0.04 8.6 ± 0.6</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Secondary (S)</td>
<td>1.8 ± 0.03 2.1 ± 0.12 3.2 ± 0.2 3.59 ± 0.04 6.1 ± 0.4</td>
<td></td>
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<tr>
<td>B. Day after sheep erythrocytes when actinomycin D† was injected (0.08 mg/kg rabbit)</td>
<td>Primary (P)</td>
<td>1 (Ser 4)‡</td>
<td>14 (Ser 6)</td>
<td>10 (Ser 12)</td>
<td></td>
</tr>
<tr>
<td>Secondary (S)</td>
<td>1 (Ser 10)</td>
<td>2 (Ser 11)</td>
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</table>

* Induction involves about the first 6 hr of the latent period.
† Actinomycin D was tested before sheep erythrocytes and 2–6 hr after the cells in series 1–3 and 7–9.
‡ Supplementary series 4A was also tested during the latent period—on day 2.
Figs. 1–6.—Graph of mean parameters for the primary hemolysin response in untreated control series P (dotted line, repeated in the six figures), and graphs of mean parameters or representative individual graphs in six series and in six groups of rabbits treated before or after an intravenous injection of sheep red blood cells (sRBC) with a sublethal dose of 0.08 mg of actinomycin D (Act D) (arrows) per kilogram rabbit. In all figures, numbers in parentheses indicate the number of rabbits in each series.

In rabbits treated with Act D before receiving sRBC versus control series P, the primary response was markedly delayed and depressed in Fig. 1 and was essentially normal in Fig. 2. In rabbits treated with Act D after receiving sRBC versus control series P, the primary response was delayed and enhanced in Figs. 3, 4, and 5, but was only slightly modified in Fig. 6.

given similarly after a 6-hr interval. This dose of the drug was fatal to 16% of the rabbits within a week. A larger total dose of 0.1 mg/kg rabbit given similarly was fatal to 60% of the rabbits within a week. Because of toxicity, seepage from the vein was avoided as far as possible.

Results. Characteristic features of the primary and secondary responses after immunization are shown in the control plots in the figures. During the secondary response as compared to the primary one, the latent period and acute rise to peak titer are shorter; peak titer is usually reached at one rather
Figs. 7–12.—Graph of mean parameters for the secondary hemolysin response in untreated control series S (dotted line, repeated in the six figures), and graphs of mean parameters or representative individual graphs in six series and in six groups of rabbits injected with sRBC and treated with Act D as in Figs. 1–6.

In rabbits treated with Act D before receiving sRBC versus control series S, the secondary response was depressed in Fig. 7 and was markedly delayed and depressed in Fig. 8. In rabbits treated with Act D after receiving sRBC versus control series S, the secondary response was delayed although not appreciably enhanced in Figs. 9 and 10, and was only slightly modified in Figs. 11 and 12.

than two rates of rise although it is not much higher, and the decline is more rapid (Table 1A). The last-mentioned parameter aids in assessing the total amount of hemolysin formed especially when peak titer is approximately normal. Thus, hemolysin declines, significantly slower than corresponding controls, represent enhanced formation. Such atypically slow declines after peak titer were designated a plateau effect by Kohn.13

Actinomycin D injected into 88 rabbits during the primary response (35 controls): Data from treated series 1–6 are illustrated in Figures 1–6, and data
from control series P are repeated in each figure. Times are shown in Table 1B. A few supplementary series will be mentioned in the text. As compared to control series P, actinomycin D acted differently when injected before (series 1–2) than when injected after immunization (series 3–6).

In series 1 (Fig. 1), actinomycin D significantly delayed and depressed the mean response when given 4 or 2 days before sheep erythrocytes. A lower peak titer (3.2 log units, \( p = 0.05 \)) was attained after a longer latent period (3.9 days, \( p < 0.001 \)) and acute rise (7.8 days, \( p = 0.002 \)). In series 2 (Fig. 2), actinomycin D injected just before sheep erythrocytes only lengthened the latent period (3.5 days, \( p = 0.04 \)). Three groups of six rabbits each, supplementary to series 1 and 2, were injected with actinomycin D 1, 2, or 4 weeks prior to the injection of the erythrocytes. The response had recovered its normal character in one week.

In series 3 (Fig. 3), actinomycin D injected during induction lengthened the latent period (3.6 days, \( p < 0.001 \)) and enhanced peak titer (3.9 log units, \( p = 0.01 \)). In series 4 the drug injected during the latent period lengthened both the latent period (3.9 days, \( p < 0.001 \)) and rise to peak titer (6.7 days, \( p = 0.04 \)) and enhanced peak titer (3.96 log units, \( p < 0.001 \)). Figure 4 shows three representative treated responses. In supplementary series 4A, the response was similarly delayed and enhanced in eight rabbits given actinomycin D on day 2. In series 5 (Fig. 5), the acute rise was lengthened and depressed for 2–4 days beginning 1–3 days after treatment and then rose to an enhanced mean peak titer (4 log units, \( p < 0.001 \)). In series 6 (Fig. 6), actinomycin D injected during the decline of hemolysin produced little change. Slight erratic rises in titer began 1–5 days after treatment. Declines after peak titer in these six series, although variable, did not yield a statistically significant plateau effect.

**Actinomycin D injected into 96 rabbits during the secondary response (29 controls):** Data from treated series 7–12 are illustrated in Figures 7–12, and data from control series S are repeated in each figure. Times are shown in Table 1B. As mentioned earlier, the secondary response was initiated 1.5–2 months after an untreated initial response. A few supplementary series will be mentioned in the text. Over-all changes of the secondary response by actinomycin D were similar to those of the primary response qualitatively, but they differed quantitatively. The following effects occurred as compared to control series S.

In series 7 (Fig. 7), actinomycin D significantly depressed peak titer (3.2 log units, \( p < 0.001 \)) although the latent period (2.1 days) and acute rise (4 days) were normal in length and the decline was slower (12.1 days, \( p = 0.006 \)). In series 8 (Fig. 8), the response was strikingly abnormal. A long latent period (3.6 days, \( p = 0.01 \)) and acute rise (7.3 days, \( p < 0.001 \)) culminated in a decreased peak titer (3.3 log units, \( p = 0.05 \)). These depressed parameters overshadowed the fact that the decline after peak titer was slower (7.3 days, \( p < 0.001 \)). Three groups of 10 rabbits each, supplementary to series 7 and 8, were injected with actinomycin D 1, 2, or 4 weeks prior to the injection of sheep erythrocytes. Although not delayed, peak titer (3.4 log units, \( p = 0.02 \)) was still depressed at 4 weeks. This slower recovery from the effects of actinomycin D as compared to the primary response is in accord with our results with 500–700 R.4
In series 9 (Fig. 9), actinomycin D injected during induction led to delayed enhancement. A longer latent period (4.2 days, \( p < 0.001 \)) and acute rise (7.6 days, \( p < 0.001 \)) were followed by a high mean peak titer (3.8 log units) and a slow but variable decline (21 days). Changes by actinomycin D on day 1 in the secondary response of series 10 (Fig. 10) resembled those on day 4 in the primary response of series 5 (Fig. 5) and reflected the faster reactivity of the secondary response. This tendency was continued in series 11 (Fig. 11 versus Fig. 6). The insignificantly enhanced peak titers in series 9 and 10 were somewhat counterbalanced by enhanced formation after peak titer as evidenced by the plateau effect. In series 12 (Fig. 12), actinomycin D injected during the decline of hemolysin only resulted in slight erratic rises in titer in four of nine responses.

**Discussion.** Precise data on the effect of actinomycin D during various stages of the immune response have only been reported for a few intervals from just before to just after primary antigenic stimulation in mice and rats.\(^4\)\(^-\)\(^9\) Treatment during full development\(^2\) of the secondary response has been neglected.\(^14\)\(^-\)\(^16\) Various-sized doses of the drug before immunization (as in Figs. 1 and 2) delayed and sometimes depressed peak titer in mice\(^9\),\(^15\)\(^-\)\(^16\) and rats,\(^16\)\(^-\)\(^19\) and large doses after immunization (as in Figs. 3 and 4) delayed and depressed the response in rats.\(^18\) Delayed enhancement was only achieved by injection of sheep erythrocytes in the middle of a course of small daily doses in mice,\(^15\) although a second moderate rise was reported by treatment during the acute rise in rats.\(^18\) These findings in rats and mice are amplified by our results in rabbits.

From our work it is clearly evident: (1) that a sublethal dose of actinomycin D, preceding the injection of sheep erythrocytes at intervals for a month, did not depress the primary response as soon (Fig. 2 versus Fig. 8) or as long (series supplementary to series 1 and 2), and (2) that a similar dose during induction or during the latent period was more effective in enhancing peak titer of the primary response (Figs. 3 and 4 versus Figs. 9 and 10), but was progressively less effective as the time of testing was deferred during the acute rise and decline of hemolysin in both responses (Figs. 5–6 and 11–12). These differences with a pharmacologic agent of known specific function\(^7\)\(^-\)\(^9\) were strikingly like those encountered with large doses of x-rays (500–700 R).\(^3\)\(^,\)\(^4\) The current results may help to explain the cytotoxic and restorative effects as reported for x-rays.\(^3\)\(^,\)\(^4\)\(^,\)\(^20\)

In order to explain our results, pertinent work by others must be mentioned. The immunologically competent initial cells or succession of cells that induce the primary response are potentially different from the activated but quiescent memory cells that accumulate in large numbers during the primary response and induce the secondary response.\(^21\)\(^-\)\(^22\) Actinomycin D primarily inhibits new messenger RNA synthesis\(^7\) during the latent period\(^8\) while only to a less extent affecting DNA synthesis,\(^7\) and in near lethal amounts results in extensive cell injury.\(^9\) Restorative agents such as nucleic acid digests have alleviated the depressing effects of x-rays\(^20\) and of actinomycin D\(^22\) under certain conditions.

Interpretation of our data in these terms leads to the following: (a) Memory cells of the secondary response appear to be more sensitive to injury (Fig. 8 versus Fig. 2) and recover more slowly (supplementary series to series 1 and 2) than initial cells of the primary response. This greater sensitivity may indicate that
the secondary response depends largely on a population of primed cells.\(^2\) In contrast, unstimulated initial cells may require extra time for antigenic processing. Moreover, in untreated controls, we suggest that initial cells play a minor role during the secondary response as compared to memory cells. Otherwise, as contrasted to the primary response, the earlier and faster rise to an approximately equal peak titer in the secondary response would be followed, not by a more abrupt decline (Table 1A), but by a less abrupt decline.

(b) During induction in both responses (Fig. 3 and 9), the latent period is lengthened by the inhibition of RNA synthesis by actinomycin D and enhancement is brought about by the release of nucleic acid degradation products because of the cytotoxic action of this drug. The same sequence occurs during the latent period of the primary response (Fig. 4) which is devoted to RNA synthesis as reported by Ortiz-Ortiz and Jaroslow.\(^2\) Explanation of the events occurring during the latent period of the secondary response belongs in the next category because activities are extremely telescoped (Table 1A) and changes in Figure 10 resemble those in Figure 5.

(c) During the acute rise and decline, DNA synthesis and cell proliferation take place. As these activities are not greatly affected by actinomycin D,\(^7\)–\(^10\) the progressively lessening changes during these stages of both responses (Fig. 5, 6, 10–12) are probably related to a transient inhibition of DNA synthesis and quick recovery.

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