STUDIES ON ANTIBODY PRODUCTION

XI. Variation in the Secondary Response as a Function of the Length of the Interval between Two Antigenic Stimuli*,‡

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The differences in the antibody response between an initial and a second dose of antigen were recognized by Solomansen and Madsen in 1896 (1). In 1921, Glenny and Südmerson (2) published a classical monograph restating them in detail. Antibody in the blood serum following the second stimulus appears sooner, its concentration rises higher, and it persists longer than after the first. It has long been part of the lore of the immunological laboratory that good secondary antibody responses are dependent on a delay between the injections, but until recently no systematic studies of this phenomenon have been made. The present experiments were carried out to discover what changes in the height of the secondary response would be brought about by systematically lengthening the interval between the two stimuli.

A number of reports of limited observations have been published. Glenny and Südmerson (2) injected two doses (one L_0 unit each) of diphtheria toxin into each of two groups of guinea pigs at an interval of 7 days or of several months. The former group formed 0.1 unit of antitoxin/ml of serum, the latter 1.0 unit. According to Glenny (3) "If the interval is long enough to allow potential immunity to develop, then the second injection acts as a secondary stimulus, and a rapid production of antitoxin follows." In 1941 Schütze (4) reported on the optimal spacing of injections of bacterial vaccines. He gave two groups of mice two injections each of *Salmonella typhi murium*, spaced either 1 or 4 weeks apart. One week after the second injection, the geometric means of the O-agglutinin titers were 2.81 and 4.6, respectively. He concluded that a significantly higher titer resulted from the 4 week interval. However, there was no difference in the resistance of the mice to challenge by living *S. typhi murium*. In a study reported by Barr and Glenny (5), 0.25 Lf of alum-precipitated diphtheria toxoid

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was injected twice into groups of 15 guinea pigs at an interval of 1, 2, or 3 months. Increasingly higher levels of antitoxin resulted as the interval was increased. Stavitsky (6) gave two intravenous injections of 40 Lf units of diphtheria toxoid to three groups of two rabbits at intervals of 1, 2, or 3 weeks. Seven days following the second injection, the average antitoxin titers were 0.01, 0.15, and 15 units, respectively. Khaustova (7) immunized guinea pigs with *Clostridium perfringens* toxoid adsorbed on aluminum hydroxide. She found that the resulting antitoxin titers were maximal when the interval between two injections was from 30 to 45 days. More recently, Khabas *et al.* (8) used intervals of 30, 60, or 90 days between two injections of diphtheria toxoid (10 Lf, adsorbed on aluminum phosphate) into three groups of 10 rabbits. On the 10th day after the second injection, the titers ranged from 1 to 2 units/ml of serum in the 30 day group, and from 15 to 20 units/ml in the other two groups. Ipsen (9) studied the effect in inbred mice of lengthening the intervals between two antigenic stimuli of purified tetanus toxoid precipitated with alum. He found an exponential increase in resistance to challenge with toxin as the interval was increased to 3 weeks. The effect of longer intervals was not investigated. He attributed this exponential increase to the multiplication of mature antibody-producing cells.

Similar studies in man have been reported for immunization against tetanus, diphtheria, typhoid, influenza, and poliomyelitis. With tetanus, Ramon and Zoeller (10) found that injections of formol toxoid given at longer intervals evoked higher antibody levels. With intervals of 15 days, 3 weeks, and 1 month, the antibody titers were 1 to 10 units, 10 to 20 units, and 100 units. Boyd (11), Marvell and Parish (12), and Peshkin (13) reported that an interval of several months resulted in greater antibody titers than an interval of 1 month.

Recently, Rauss *et al.* (14) studied antibody titers to tetanus in adults, using a combined tetanus-typhoid-paratyphoid-dysentery vaccine. With injection intervals of 4, 8, and 12 weeks, the antibody titers were 1.53, 3.5, and 3.7 IU per ml, respectively. According to this author, maximum titers are attained at 8 weeks; no further increase in titer occurred with prolongation of the interval. In a routine study of diphtheria toxoid of low alum content in several groups of children and adults, Horner *et al.* (15) found that the antitoxin titer was increased by lengthening the interval between doses of toxoid from 4 to 8 weeks.

Felix *et al.* (16) measured the antibody response to typhoid vaccine in two groups of twenty adult female nurses. Typhoid agglutinins were higher with a 3 week interval than a 2 week one.

Mogabgab *et al.* (17) studied the response to two injections of polyvalent influenza vaccine given at intervals of 6, 8, or 10 weeks. They were unable to demonstrate an appreciable difference in the titers.

Finally, MacLeod *et al.* (18) measured the antibody response to Salk-type poliomyelitis vaccine, and concluded that an interval of at least 4 weeks between injections was necessary for an adequate response.

Several problems obscure the interpretation of these data. Many experimenters used precipitated antigens, the slow release of which doubtless caused continuing stimulation by the first dose of antigen. When relatively pure fluid antigen was used, the intervals studied were often too short to permit conclusions about the optimal interval for a maximum secondary response. In

the human studies, previous contact with the antigens used could not be ruled out and, indeed, often complicated the interpretation of the results.

In this communication the effect of increasing the length of the interval between the first and second doses of diphtheria toxoid upon the peak secondary antibody response in mice is described. Large numbers of animals were used in the hope of reducing the significance of individual animal variation in response to antigenic stimulation.

It will be shown that, in the system described, the height of the secondary antibody response increased as the interval between antigen injections was lengthened, but only up to 40 days. Intervals greater than 40 days did not result in antibody levels higher than those at the 40 day interval.

Materials and Methods

Animals.—Two hundred and sixty white male mice, 7 weeks old, weighing between 18 and 20 gm, were fed Purina lab chow and given free access to water. Nine groups of ten mice each were used to study the primary response and 17 groups of ten mice each were used to study the secondary response.

Antigen.—Purified diphtheria toxoid containing 1875 Lf per ml was kindly supplied by the Division of Biologic Laboratories of the Commonwealth of Massachusetts. Dilution was made with sterile saline to a final concentration of 10 Lf per 0.5 ml, and this amount was injected subcutaneously between the scapulae.

Antisera.—Mice were bled from the tail; the sera obtained were stored at −20°C until use. Antibody titers were measured by hemagglutination of tannic acid–treated, diphtheria toxoid–sensitized cells (19). Titers were expressed as the logarithm to the base of 2 of the reciprocal of ten times the highest serum dilution containing detectable antibody. Thus, serum dilutions of 1/20, 1/40, 1/80 etc. correspond to titers of 1, 2, 3, etc. Since serum dilutions of less than 1/20 were not tested for hemagglutinating antibody in any of the experiments described, reference to non-responding mice means that such mice failed to produce antibody in serum dilutions of 1/20 or greater.

RESULTS

The Primary Response.—Fig. 1 indicates the responses made by nine groups of ten mice each to a single subcutaneous injection of 10 Lf of diphtheria toxoid.

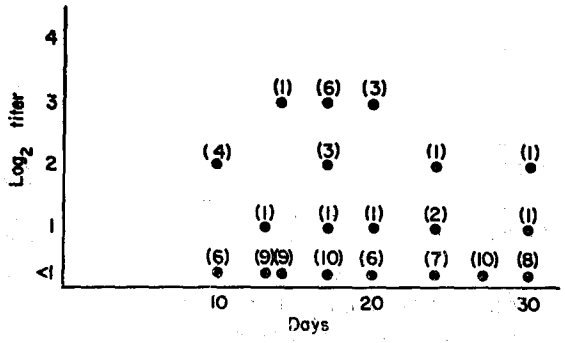

FIG. 1. Primary response in mice to 10 Lf fluid diphtheria toxoid, given at day 0. Numerals in parentheses indicate numbers of mice at each point.

Only 25 of the 90 mice formed antibody at our level of detection. Those which did respond evidently carried an antibody titer for 2 weeks or more, from the 10th through about the 24th day. The two groups tested for antibody 17 days after a single injection were atypical: in one group of ten there were no responses; in the other group of ten all responded.

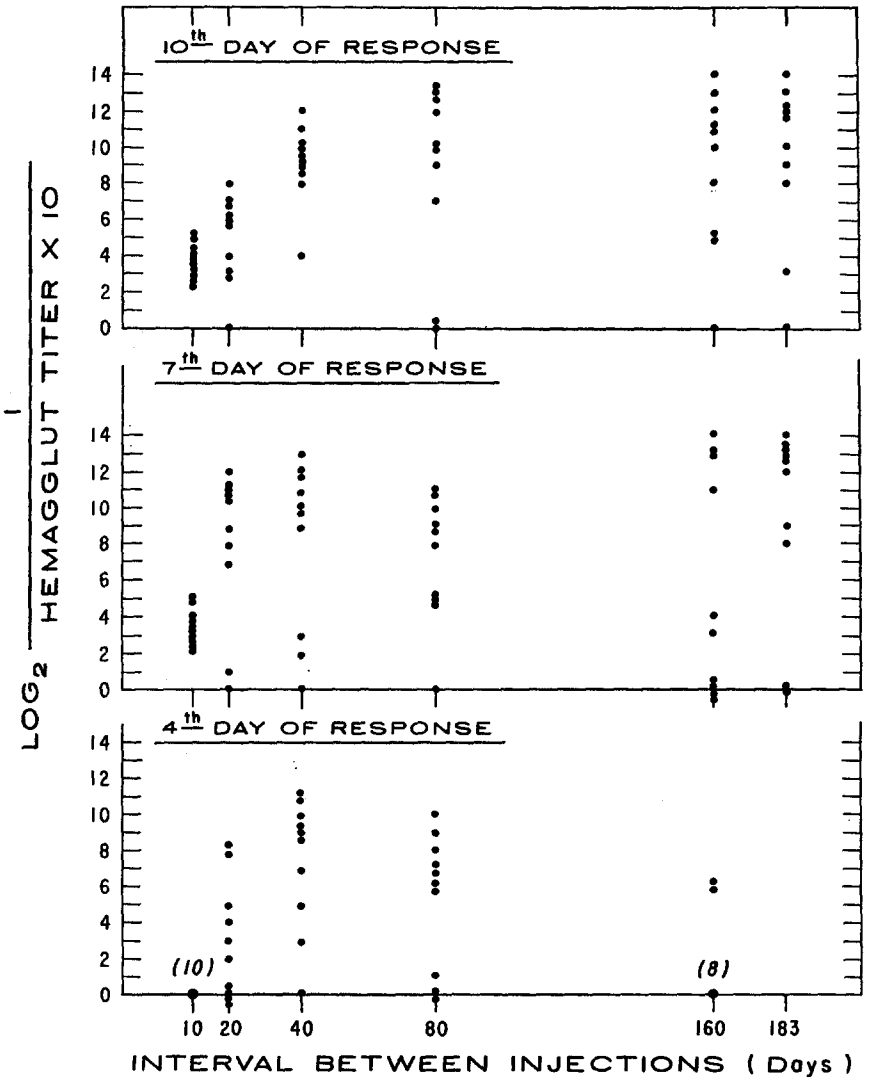

FIG. 2. Relation of interval between diphtheria toxoid injections and height of the resultant secondary response. Each dot represents one mouse. Double dose antigen at 183 day interval.

Clearly, 10 Lf of fluid diphtheria toxoid is an indifferent overt stimulus to mice, producing responses in only 28 per cent. However, as the following experiments indicate, it is an effective "priming" dosage.

The Secondary Response.—In this investigation of the effect of the duration of the interval between two injections on the peak of the subsequent antibody response five groups of 30 mice each and one group of 20 mice were used. These 170 mice were given a single dose of 10 Lf of fluid diphtheria toxoid on

TABLE I

Secondary Response to Diphtheria Toxoid in Mice

Interval between 2nd stimulus and bleeding	Method of estimation	Intervals between stimuli in days					
		10	20	40	80	160	183*
days							
4	Avg. titer	40	144	6952	2178	128	
	6th mouse titer	40	80	5120	1280	20	
	Log_2 avg. titer	1	3.6	7.5	5.6	2.0	
7	Avg. titer	144	13,186	20,964	6,496	34,000	105,512‡
	6th mouse titer	80	20,480	10,240	5,120	20,480	163,840
	Log_2 avg. titer	3.6	9.2	8.3	7.4	6.2	10.3
10	Avg. titer	160	736	11,024	31,364	37,440	77,328
	6th mouse titer	320	1,280	10,240	10,240	20,480	81,920
	Log_2 avg. titer	4.3	6.8	9.2	8.9	10.3	10.3

Mice received two doses subcutaneously of purified fluid diphtheria toxoid (D) 10 Lf per mouse on day 0, and on day indicated.

* These two groups received 20 Lf D as a secondary stimulus.

‡ Titers of pooled sera of 183rd day group: 7th day, 80,000; 10th day, 80,000.

day 0. A subsequent dose was given each group on day 10, 20, 40, 80, 160, or 183 respectively. Of each group, ten were bled and killed on the 4th, 7th, or 10th day after they had received the second injection. This experimental plan resulted in 17 groups of ten mice each.

The results of the antitoxin titration are shown in Fig. 2 and summarized in Table I. There was an increase in the maximum potential antitoxin response during the first 3 to 6 weeks following a first injection of toxoid. Thereafter, as the interval was lengthened, there was little additional increase, although the maximum was maintained, and perhaps increased slightly depending on how the data are summarized. The average peak titer seems to be dose-dependent at this dose level, because the titer increased when the dose was doubled (to 20 Lf) in the groups injected on the 183rd day.

The data in the table are summarized in three different ways: as the average

titer, assigning a value of 1/20 to those mice which did not respond; as the median mouse (6th) in the group; and as the average number of the tubes at the end-point in the series of doubling dilutions beginning the count at 1/20. The average titer and that of the 6th mouse show close agreement, especially if it is borne in mind that the end-point varies by a factor of 2 in such a titration. The $\log_2$ expression tends to damp both the high and the low titers in a group, but it particularly minimizes the effect of one or two high titers.

An interesting comparison between mathematical and physical pooling is available for the 183rd day group. Serum in equal amount (0.1 ml) from each of the ten mice in each subgroup was pooled, and the two pools titrated. The titer of each pool was 1/80,000, a value in good agreement with the average titers of 1/106,000 and 92,000.

DISCUSSION

This experiment was designed to determine whether an increasingly effective potential for the synthesis of antibody developed with the passage of time after a first exposure to antigen. Clearly, there is an increase for the first 40 days, but little further increase thereafter. These results confirm those summarized in the introductory statements, and, taken with these, provide suggestive evidence that the increased height of the secondary response is not due to a steady, *intrinsically* determined multiplication of sensitized cells between the injections. If it is due to cell multiplication, the stimulus to multiplication must be connected with the presence of adequate amounts of antigen. Moreover, the attainment thereafter of a relatively steady state of sensitization or priming suggests that, if a specific cell population is involved, it must have reached an equilibrium such that new cells arise only as old ones die, or that some factor has been diluted to the point where it is transmitted to only one daughter cell at each division, or that the population does not divide at all until a new stimulus reaches it.

The group with high responses on the 183rd day received 20 Lf, twice the dose administered to other groups. The resulting antibody titers were significantly higher, probably indicating that the 10 Lf dose of antigen was not a saturating one. In the experiments reported in the accompanying papers, a dose of 20 Lf was given on two occasions, 40 days apart; this procedure resulted in a reproducible secondary response which was used as a standard.

The response of this strain of mice to a single dose of fluid diphtheria toxoid was poor, only 25 of 90 showing antibody by hemagglutination at the 1/20 level, the lowest titer tested. Two weeks or more after an initial injection of antigen, about 90 per cent of mice responded to a second injection. No explanation for the failure of one in ten is available; it is a regular phenomenon in immunization.

Of the three methods of striking averages, there is better agreement between the arithmetic mean and the median, than between these and the average $\log_2$ titer. In the two instances where actual pooling was compared with mathematical pooling, the values show good agreement. However, both these methods are greatly influenced by a single high response, while the $\log_2$ titer minimizes such an effect, but magnifies that of the poor responders.

SUMMARY

As the interval between two antigenic stimuli to mice is lengthened, the magnitude of the response to the second dose increases up to approximately 40 days; thereafter it remains at a fairly constant, high level.

We are grateful to Dr. Maria C. Michaelides and Dr. Eli Sercarz for carrying out some of the hemagglutination tests.

APPENDIX

Statistical analysis of the results was kindly performed by Dr. David W. Alling, National Institute of Allergy and Infectious Diseases, Bethesda.

The Wilcoxon two sample test was used in comparison of the antibody titers of one interval between injection with the antibody titers of succeeding intervals between injections. The results of two-sided tests (titers tending to change with increased interval *vs.* titers not tending to change with increased interval) are presented in Table II.

TABLE II

Wilcoxon Two Sample Test Comparing Antibody Titers of One Injection Interval to Succeeding Intervals

Days of secondary response	Responses at pairs of intervals compared; significance level of difference				
	10, 20	20, 40	40, 80	80, 160	160, 183
4	$p < 0.05$	$p < 0.02$	$p > 0.10$	$p < 0.02$	—
7	$p < 0.05$	$p > 0.10$	$p > 0.10$	$p > 0.10$	$p > 0.10$
10	$p > 0.10$	$p < 0.002$	$p > 0.10$	$p > 0.10$	$p > 0.10$

For the 4th day of the secondary response, the progressive increase in height of the response with successive intervals was significant up to the interval of 40 days.

In analysis of the 7th day of the secondary response, the height of the antibody response for the 20 day interval was significantly greater than that for the 10 day interval. However, for all succeeding intervals after the 20 day interval, there was no significant change in the height of the antibody titers.

On the 10th day of the secondary response, in comparing the injection intervals of 10 and 20 days, there was no significant difference in the antibody titers. The change, or increase, in titers between the 20 and 40 day injection intervals was significant, but beyond the 40 day interval there was no significant change in the height of the antibody titers.

In summary, the analysis shows that increasing the injection interval from 20 to 40 days caused a significant change, or increase, in titer on the 4th and 10th day of the response, but not on the 7th day. The 7-day titers reached a maximum with a 20 day interval. Increasing the interval between injections from 40 to 80 days failed to cause significant change in the antibody titer in all instances. Therefore, maximum antibody production was reached when intervals between injections were not less than 20 days; beyond 40 days a plateau was reached.

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