Antigens in Immunity

VII. ANALYSIS OF IMMUNOLOGICAL MEMORY

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Summary. Rats were given primary and secondary injections in saline solution without adjuvants, of *Salmonella* flagellar antigens, the dose varying from 10 pg $(10^{-11}g)$ to 1 mg. Rats were bled at various times after injection, and levels of both total (7S+19S) and mercaptoethanol resistant (7S) anti-H antibody were determined, using a serial two-fold dilution assay. The data from several thousand such titrations was entered on IBM punched cards and a series of programs were written to allow computer analysis thereof.

The chief findings which emerged from the study were:

(1) Important differences exist in the kinetics of primary and secondary responses.

(2) The excess antibody formation characteristic of the secondary response is essentially a short-lived phenomenon.

(3) Conditions may be defined for the demonstration of excellent secondary responses where the additional antibody formed is solely 19S.

(4) Secondary responses are evoked only when the second dose of antigen equals, or preferably exceeds, the first dose.

(5) Peak antibody titre: antigen dose curves differed for primary and secondary responses.

(6) Memory can be quantitated in a variety of ways. The hypothesis that memory stems from a series of cellular events induced by antigen but independent of actual antibody formation is discussed.

(7) Optimal conditions for the study of the role of antigen in the secondary response were established.

INTRODUCTION

The phenomenon of immunological memory has been known for over 40 years (Glenny and Südmersen, 1921). Since then, numerous reviews have dealt with the topic and its implications for cellular immunology (Burnet, 1941; Taliaferro and Taliaferro, 1952; Jerne, 1960; Nossal, 1962; Uhr and Finkelstein, 1963). We would have hesitated to add to this already voluminous literature, were it not for three considerations:

(1) We are engaged in a critical survey of the role of antigens in the induction of immunity and tolerance (Ada, Nossal and Pye, 1964a; Ada, Nossal, Pye and Abbot, 1964b; Nossal, Ada and Austin, 1964a, b; Nossal and Ada, 1964; Ada, Nossal and Austin, 1965). This study involves the injection into rats of flagellar antigens labelled with radioactive iodine, under carefully defined conditions. For adequate study of the induction of memory, it was thus essential to determine the kinetics of the secondary response in our system, and particularly the optimal combination of antigen doses.

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(2) Much of the work on the secondary response was done before the distinction between the 7S and 19S antibody responses had emerged clearly. In a number of systems secondary 19S responses were not obtained (Uhr and Finkelstein, 1963; Svehag and Mandel, 1964), but preliminary experiments from our laboratory (Nossal, Szenberg, Ada and Austin, 1964) revealed circumstances where excellent '19S memory' could be obtained. This point seemed worthy of further study.

(3) The extraordinary individual variation in immune responses, particularly with small antigen doses, is well known. In a sense, each animal injected with antigen represents an experiment in its own right. Accordingly, exhaustive statistical treatment of antibody titration results is necessary. These difficulties may have limited the degree of variation in dosage combinations used in previous studies of the secondary response. With the use of computer techniques, statistical analysis has become much less tedious.

Over the past 2 years, we have performed some 10,000 anti-H antibody titrations on serum samples from over 2000 rats injected with *Salmonella* flagellar antigens, as part of many different experiments. Most of these animals were bled only once, at the time of killing, and were thus not useful for kinetic analysis. However, some 400 were bled repeatedly, as often as seventeen times. We felt it would be of interest to use computer techniques to extract the most useful correlations from this otherwise rather unmanagable mass of information. The present paper is an attempt to summarize the salient findings of the study.

MATERIALS AND METHODS

Animals

Randomly-bred Wistar albino rats of both sexes, aged 12 weeks at first immunization, were used. They were fed on 'Barastoc' dog cubes, cabbage and tap water.

Antigen injections

The antigens used were intact flagella and soluble flagellin from Salmonella adelaide, prepared as previously described (Ada *et al.*, 1964b). All injections were given into both hind foot-pads in saline solution without adjuvants. Doses are expressed as mg, μ g, ng (nanograms, 10^{-9} g), and pg (picograms, 10^{-12} g), and refer to the total dose. Secondary injections were given 6 weeks after the primary injections.

Serum antibody titrations

Serum anti-H antibody was titrated by a serial two-fold dilution assay based on immobilization of motile *Salmonella* organisms, described in detail previously (Ada *et al.*, 1964b). Both total (7S+19S) antibody and 'mercaptoethanol resistant' (MER or 7S) antibody were measured (Nossal *et al.*, 1964, 1964a).

Computer techniques

All data was entered on IBM punched cards by the Scientific Computing Centre of IBM (Australia) Pty Ltd. One of us (G.N.) wrote three programs in FORTRAN IV to handle the data, and is grateful to Mr J. Pinn (IBM) and Mr J. Pye (Hall Institute) for helpful criticism. Computations were done on the University of Melbourne's IBM 7044 digital computer.

For the first program, SECRESP 1, data were arranged in two separate ways. Firstly, rats were grouped according to the primary injections they had received, regardless of the nature of the secondary injection, if any. These were quite large groups (e.g. fifty-one

rats for 100 pg of flagella; forty-one rats for 100 ng of flagella), and thus reliable information on primary response kinetics was obtained. Secondly, rats were grouped according to both primary and secondary injections, resulting in a larger number of small groups (three to nine animals). Thus, information on secondary response kinetics was somewhat more subject to sampling errors. This may account for certain deviant points in the graphs which follow.

The program SECRESP 1 returned the following information for each of eighteen set time points in the primary and secondary responses: geometric mean titre for each time point; geometric mean peak 7 day (peak 19S) primary titre separately for males and females in each group; geometric mean 6 week (near-peak 7S) titre, separately for males and females; the number of animals bled at each time point; the standard deviation (in \log_{10}), the coefficient of variation; maximum and minimum titres for each time point; all of the above was calculated for both total and MER antibody; finally, the ratio of mean MER to mean total antibody was calculated for each time point.

In calculating secondary response kinetics, it was important to know, in detail, what would have been the characteristics of the primary response antibody curve between 6 weeks (the time at which all secondary injections were given) and 10 weeks (the last bleed performed in secondary response rats). For example, if a four-fold rise in titre had been the rule over this period, even *without* any secondary injection, misleading conclusions as to the efficacy of a secondary injection might have been reached had one presumed that the primary response curve was a plateau over this period. Accordingly data from eightyseven rats given a primary injection only, and bled frequently after 6 weeks, were used as input for the program SECRESP 2. It returned factors giving the slopes of the antibody curves between 6 and 8, and 8 and 10 weeks, calculated separately for each antigen dose.

The program SECRESP 3 was somewhat more complex. It used as input both the mean results derived from SECRESP 1 and SECRESP 2, and the original titration data from individual rats. Data were arranged as for the second part of SECRESP 1, namely according to both primary and secondary injections. The output returned by this program is described below.

Let primary titres = PT

Let secondary titres = ST

Let geometric mean titres = \overline{PT} or \overline{ST}

Let PT_b denote *primary* titres expected following a given dose in situations where that dose had in fact been used as a secondary stimulus.

Then, various values can be calculated.

(1) The net secondary titres (NST). These were calculated for each time point of each rat, and subsequently geometric means and standard deviations were calculated for each group of rats stimulated with a given primary and secondary dose. This applies also to the other values described below. The NST is simply the ST less the PT which that animal would have had, had it received no secondary injection. The value subtracted was the PT at 6 weeks multiplied by a factor dependent on the slope of the expected primary response curve beyond the 6 week point. Thus

$$NST = ST - PT$$

(2) The excess net secondary titres (ENST). Obviously, the NST alone does not tell us whether an animal is exhibiting specific secondary responsiveness or 'memory'. Memory is only present when the NST is greater than \overline{PT}_b , the mean primary titre to be expected

from the dose used as a secondary stimulus. However, if we subtract \overline{PT}_b from the NST, we have one measure of memory, namely the number of titre units by which the NST is in excess of what might have been expected in the absence of memory. Thus

$$ENST = NST - \overline{PT}_b = ST - (PT + \overline{PT}_b)$$

(3) The adjusted excess net secondary titres (AENST). This concept is predicated on the hypothesis that the way in which an animal responds to a primary injection of antigen is of predictive value for the secondary response. Thus, animals giving below-average titres after the first injection might respond less well to a secondary stimulus than those giving above-average titres. Accordingly, the excess net secondary titres of each rat were adjusted by a factor representing the mean 6 week primary titre expected for that groups' primary injection divided by the actual 6 week primary titre achieved by that rat. For example, if a rat had responded twice as well as others in the group to a primary injection, all excess net secondary titres from that rat were multiplied by one-half. The differences between the excess net secondary response and the adjusted excess net secondary responses were usually minor, but for the sake of consistency, we have plotted the adjusted value in all the graphs which follow. Thus,

$$AENST = ENST \times \frac{\overline{PT}}{PT} = ST - (PT + \overline{PT}_{b}) \times \frac{\overline{PT}}{PT}$$

(4) Memory factor (MF). The memory factor is the net secondary titre divided by the mean primary titre which that particular secondary antigen dose would have elicited at each particular time point. It thus measures how many times greater the secondary response was than a primary would have been, and is perhaps the best available quantitative measure of memory. Memory factors were calculated for 4, 7 and 28 days after secondary injection. Thus,

$$\mathrm{MF} = \frac{\mathrm{NST}}{\overline{\mathrm{PT}}_{\mathrm{b}}} = \frac{\mathrm{ST} - \mathrm{PT}}{\overline{\mathrm{PT}}_{\mathrm{b}}}$$

In one set of circumstances, the memory factor proved to be a misleading value. When the 6 week primary titre was high and the secondary response poor or absent, random variations of $\frac{1}{2}$ to 1 log₂ in titration results sometimes led to spuriously high memory factors, particularly with small secondary doses. To overcome this difficulty, an additional set of values was calculated namely the bumping factors.

(5) Bumping factors (BF). These represent simply the actual secondary titres divided by the actual 6 week primary titre given by the animal. Thus,

$$BF = \frac{ST}{PT \ 6 \ weeks}$$

The main usefulness of the bumping factors was in providing a check on the validity of the memory factor. Take, for example, a rat having a 6 week primary titre of 800 and a 4 day secondary titre of 1200. This difference could well be due to titration error. Suppose the \overline{PT}_{b} at 4 days with the antigen dose used had been 10. A memory factor of 40 would have resulted; but the program was so written that the low bumping factor of 1.5 would have identified the doubtful significance of the apparently high memory factor value.

(6) Assessment of presence of secondary response. The program determined for each rat whether it obeyed certain criteria laid down for presence or absence of a secondary

response. To qualify as positive, each rat had to pass a series of tests determining whether it had given significantly large memory factors *and* bumping factors. Though this procedure was unnecessary in most cases where both primary and secondary antigen doses were large, and the secondary response quite obvious, it proved useful in more borderline cases where either the primary or the secondary dose was small.

The program SECRESP 3 consisted of about 270 source statement cards and gave rise to some 700 internal statements. It is therefore too long to reproduce here but copies can be furnished on request. The 7044 computer compiled the above program, entered it into storage, and processed forty-two groups of data in 4 minutes.

RESULTS

SERUM ANTIBODY CURVES IN PRIMARY AND SECONDARY RESPONSE

Fig. 1 shows titres of total and MER antibody of rats that had received primary, or primary and secondary, immunization with 100 μ g of flagella. This is one of numerous graphs which could equally well have been shown and is presented here as a typical result plotted in the conventional manner. The two curves for total antibody resemble each other in that both exhibit a lag period followed by a sharp rise. In the primary response, this is followed by a brief plateau and a prolonged slow rise. In the secondary response, the titres fall slowly but steadily after the peak at 6 days. Moreover, the curves for MER (7S) antibody show marked differences. In the primary response, most antibody formed in the first 7 days is 19S, whereas in the secondary, there is a strong early 7S component.

The remaining results presented in this paper are from data similar to that given in Fig. 1 analysed in the manner outlined in 'Materials and Methods'.



FIG. 1. Total and ME-resistant serum antibody titres following primary and secondary injection of 100 µg of flagella. Vertical bars represent standard deviations. $\times ---- \times$, Secondary response, total antibody; $\times --- \times$, secondary response, mercaptoethanol resistant antibody; $\bullet --- \bullet$, primary response, mercaptoethanol resistant antibody.

EXCESS ANTIBODY MANUFACTURED BECAUSE MEMORY IS PRESENT

Fig. 2 shows the $\overline{\text{AENST}}$ of rats given two doses of flagella 6 weeks apart, each group receiving the same dose as primary and secondary stimuli, i.e. 10 ng and 10 ng, 100 ng and 100 ng up to 100 µg and 100 µg. Standard deviations are omitted from this and subsequent graphs for the sake of clarity. The AENST is a reflection of the antibody levels in excess of what would have been expected if the primary and secondary injections had each stimulated an equal amount of antibody formation.



FIG. 2. Excess total antibody formed in the secondary response to flagella. The doses quoted for the various groups refer to both primary and secondary stimuli. For explanation of 'adjusted excess net secondary titre' see Materials and Methods. Antigen doses (same dose 1 and 2): \Box , 100 µg; \times , 10 µg; \circ , 1 µg; \bullet , 100 ng; \blacktriangle , 10 ng.

Three points emerge clearly from this graph. Firstly, the period of production of 'excess' antibody is relatively short (4 weeks or less). After this time, rats showed no more (and sometimes less) antibody than might have been expected on the basis of a simple summation of two identical responses. This shows a striking difference in kinetics between antibody production in a secondary as compared to a primary response. In the latter, antibody production is very prolonged (Nossal *et al.*, 1964a). Secondly, the rate of appearance of excess antibody is dependent on the dose, being more rapid with larger doses. Thirdly, the total amount of excess antibody produced varies with dosage over the whole range tested. In all cases, maximum titres were achieved around 6-7 days.

production of 19S and 7S antibody in primary and secondary response dose-response relationships

It has been shown that 'excess' antibody was produced in a secondary response to flagella and that the kinetics of this response differed from that of a primary response. The results were now analysed to see to what extent 19S and 7S components contributed to this difference. In Fig. 3, dose response relationships of total and 7S antibody are presented. The top two curves show the titres at 6 weeks in the primary response, and represent peak or nearpeak values. The lower two curves show the 7 day primary titres (total and MER) and represent the peak of the 19S phase. In this system, reductions in titre of $0.5 \log_2$ are common after ME-treatment even with authentic 7S antibody fractions (Nossal *et al.*, 1964) so that it is clear that with all doses the antibody by 6 weeks is predominantly, if not solely, 7S. The peak level of 7S shows a steep rise between 10 ng and 100 ng and little rise over the next 3 log₁₀ steps. These more extensive results have thus confirmed our earlier statement (Nossal *et al.*, 1964a) that 100 ng of flagella approaches the maximally immunogenic dose as regards 7S antibody. In contrast to the findings for antibody at 6 weeks, most of the antibody found at 7 days, with all doses tested, was 19S (lower two curves, Fig. 3). The level of 19S antibody and the proportion of 19S:7S antibody increase over the range 10 ng to 100 µg.



FIG. 3. Dose-response curves for total and MER antibody in the primary response to flagella. The 6 week curves represent the peak or near-peak of the 7S phase; the 7 day curves represent the peak of the 19S phase. \bigcirc , Total antibody, 6 weeks; \bigcirc , mercaptoethanol resistant antibody, 6 weeks; \triangle , total antibody, 7 days; \blacktriangle , mercaptoethanol resistant antibody 7 days.

Dose-response relationships for AENST are given in Fig. 4. The values at 7 days (peak or near peak) in the secondary response were plotted against dose of flagella. In each case, the same dose was used for primary and secondary immunization. The top curve is total antibody and thus is an extension of some results given in Fig. 2. With the exception of one value, there is an increase in excess antibody produced with increasing dosages. With small doses, the excess antibody, though small in amount, is wholly MES in character. Previous work (Nossal *et al.*, 1964) proved that such antibody did indeed have a sedimentation coefficient of approximately 19S. With larger doses, substantial amounts of excess 7S antibody are produced, the proportion of 19S to 7S varying rather widely from rat to rat and group to group. These results thus show a dose-response pattern which differs markedly from that seen for peak titres in the primary response. The 7 day secondary response pattern, though composed of both 19S and 7S components, is rather similar in character to the 7 day primary response pattern, which is largely 19S in nature.

FURTHER ANALYSIS OF THE 19S COMPONENT IN IMMUNOLOGICAL MEMORY

The above results indicate clearly that 'excess' antibody formation in the secondary response, i.e. antibody formed due to the condition of memory, can be composed of 19S, or 7S and 19S components. As memory for 19S antibody had not previously been demonstrated in other systems, this aspect was further analysed.



FIG. 4. Dose-response curves for total and MER antibody in the secondary response to flagella. The doses quoted on the abscissa refer to both primary and secondary stimuli. All results refer to samples taken 7 days after secondary injection and represent the peak values. \triangle , Total antibody; \blacktriangle , mercapto-ethanol resistant antibody.



FIG. 5. Demonstration of 19S secondary responses. The graphs on the left refer to rats given 1 ng of flagella as primary stimulus and 10 µg of flagella as secondary. The graphs on the right refer to rats given 10 µg of monomeric flagellin as both primary and secondary stimuli. Note that ME treatment substantially reduces the titres in the first 7 days. \triangle , Total antibody; \blacktriangle , mercaptoethanol resistant antibody.

In a previous communication (Nossal *et al.*, 1964) we reported preliminarily that the best way to demonstrate a secondary 19S response was to use a small, sub-immunogenic priming dose and a larger secondary dose. Further work has amply confirmed this finding, and a typical experiment is shown on the left-hand side of Fig. 5. Rats in this group had received 1 ng of flagella as the primary stimulus, which had not resulted in detectable antibody formation, and a secondary dose of 10 μ g of flagella. A brisk secondary response, almost exclusively of ME-sensitive antibody ensued. Gradient density ultracentrifugation studies (Nossal *et al.*, 1964) proved that this was indeed 19S antibody. Another clear-cut way of demonstrating a secondary 19S response was to use monomeric flagellin as a secondary stimulus. Previous work has shown that in doses under 100 μ g, primary foot-pad injections of flagellin caused no early 19S response. On the right hand side of Fig. 5, the adjusted excess net secondary titres for a group of rats given 10 μ g of flagellin both as a primary and a secondary dose are shown. Though the primary injection had failed completely to give 19S antibody, the secondary dose resulted in a brisk response. During the first 7 days, this was predominantly ME-sensitive antibody, but by 2 weeks most of the excess antibody was ME-resistant.

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LISTING OF	ANTIGEN-COMBINATIONS	LEADING	то	CLEAR-CUT

Primary stimulus	Secondary stimulus
1 ng flagella	1 ng flagella
100 pg flagella	100 ng flagella
1 ng flagella	100 ng flagella
10 ng flagella	100 ng flagella
100 ng flagella	1 ug flagella
100 ng flagella	1 ug flagella
1 µg flagella	1 ug flagella
100 pg flagella	10 μg flagella
1 ng flagella	10 μg flagella
100 ng flagella	10 μg flagella
1 ng flagellin	10 μg flagella
10 ng flagellin	10 μg flagella
10 ng flagellin	10 μg flagella
100 ng flagella	10 μg flagellin
100 ng flagella	1 μg flagellin
1 ug flagella	1 μg flagellin
10 ug flagella	10 μg flagellin
10 ug flagellin	10 μg flagellin

In Table 1, all the combinations of antigen doses which resulted in distinct '19S memory' are listed. In all cases, the 19S antibody appeared early, peaking at 4–7 days, and then rapidly disappeared. Most of the groups had received small primary doses and larger secondary ones. It is quite possible that animals receiving large primary *and* secondary doses (e.g. 100 μ g of flagella) might have produced substantial amounts of 19S antibody in the secondary response. However, this might have been 'masked' by the brisk 7S responses induced under these circumstances. We have, as yet, no good method of titrating 19S antibody in the presence of an excess of 7S antibody.

THE QUANTITATION OF IMMUNOLOGICAL MEMORY

Detailed study of the quantitation of memory is complicated by the fact that at least three variables are involved, namely the primary stimulus (which 'induces' the memory); the secondary stimulus (which 'evokes' it); and, as there are kinetic differences between the two responses, the time after antigen injection at which the determinations are made. In Fig. 6, three curves are plotted, each showing the adjusted excess net secondary titres achieved following a *constant* primary antigen dose and 7 days after a *variable* secondary dose. The dotted line refers to rats given 100 pg of flagella as a primary, and 100 pg to 10 μ g as a secondary, stimulus. The solid curve represents rats given 100 ng as a primary and the broken curve represents rats given 100 μ g as a primary. The results show that when the primary dose was only 100 pg, all groups given a secondary dose from 1 ng upward showed some excess antibody, i.e. some 'memory'. When a thousand-fold larger priming dose was given, rats given 1 ng and 10 ng showed little memory; and when the priming dose was a thousand-fold larger again, even 10 μ g of flagella as a secondary dose evoked no excess antibody. It is thus clear that to *evoke* an anamnestic response in this system, the second dose of antigen must be as large as, and preferably larger than, the primary dose.



FIG. 6. The importance of the antigen dose used to *evoke* responses. The doses on the abscissa refer to the secondary stimulus with flagella. With a primary dose of 100 pg of flagella, all secondary doses > 100 pg demonstrate a slight degree of 'memory'. With a primary dose of 100 ng, memory is unimpressive unless the secondary dose is 1 µg or more. With a primary dose of 100 µg, no secondary dose below 100 µg evokes memory. ×, Primary dose, 100 pg; \bigcirc , primary dose, 100 ng; ⊕, primary dose, 100 µg.

This analysis has been extended to the situation where rats given variable doses of antigen as a primary stimulus were then given a constant dose of antigen as a secondary stimulus. Fig. 7 shows the results when the primary dose varied from 10 pg to 100 μ g and the second dose was usually 10 μ g (100 μ g for the last point). The 'amount of memory' is plotted against primary antigen dose, the top curve giving AENST and the lower curve representing the memory factors at 7 days. The following conclusions can be drawn.

(a) Over a wide range of primary antigen doses, the 'amount of memory' induced is antigen dose-dependent.

(b) Sub-immunogenic primary doses (i.e. doses <10 ng) can induce significant memory provided that the secondary stimulus is substantially larger. This was well demonstrated also with other secondary doses (Table 1).

(c) It was shown in Fig. 4 that, in contrast to peak primary response titres, $\overline{\text{AENST}}$ showed a steep rise over the antigen dose range 100 ng to 100 µg. However, analysis of this result was complicated by the fact that both primary and secondary doses were increased. The more stringent measure of the 'amount of memory', the memory factor, shows a similar increase between 100 ng and 10 µg, even though the secondary dose was constant at 10 µg.



FIG. 7. Quantitation of memory. For explanation of memory factors, see 'Materials and Methods'. The doses on the abscissa refer to primary stimulation with flagella. \times , Adjusted excess net secondary titre 7 days after a secondary injection, with variable primary doses (see text); \bigcirc , memory factors for varying primary doses (see text).

Table	2
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COEFFICIENT OF VARIATION OF 6 WEEK PRIMARY TOTAL ANTIBODY TITRES OF VARIOUS GROUPS OF RATS

Dose	Flagella	Flagellin
100 pg	284	0*
1 ng	237	91
10 ng	107	28
100 ng	17	60
1 µg	7	21
10 µg	13	38
100 µg	9	80
1 mg	N.D.†	13

*No animals with positive titre.

[†]Only two animals in this group.

INDIVIDUAL VARIATION IN IMMUNE RESPONSES

As part of the program SECRESP 1, the coefficient of variation $(S.D. \times 100 \div Mean)$ of the various groups was calculated. This index of the degree of individual variation is

tabulated for the 6 week primary titres in Table 2, which shows that results with flagella are extremely variable below 100 ng (the lowest uniformly immunogenic dose), but relatively consistent at or above this dose. By contrast, results with monomeric flagellin show a marked degree of individual variation over the whole range 1 ng to 100 μ g.

DOSE-RESPONSE RELATIONSHIPS USING FLAGELLIN

The variability of results with flagellin makes it difficult to construct dose-response curves for this antigen. Fig. 8 presents a proportion of the results obtained with flagellin, and it can be seen at once that it is more difficult to draw lines of best fit than with the flagella results. The lowest curve represents the mean 6 week primary titres of total antibody. The most striking difference between this curve and the flagella dose-response curve (Fig. 3) is the extremely flat nature of the curve with antigen doses above 1 ng. Even a ten-thousand-fold increase in dose causes relatively little increase in mean peak titre. The dose-response curve for adjusted excess net secondary response at 7 days for groups of animals given the same dose of flagellin as primary and secondary stimulus is represented by the middle line. Again, the slope of this curve is lower than with flagella (Fig. 4). Finally, the top curve shows the 'amount of memory' induced by various doses of flagellin as judged by the 7-day excess net secondary titres evoked by a constant secondary dose of 10 μ g of flagella. This curve is thus directly comparable with the upper curve of Fig. 7. Sub-immunogenic doses of flagellin can induce memory, and this is particularly well shown when a large dose is given as the secondary injection. In the lower dose ranges, the 'amount of memory' induced is equal to or greater than that following similar primary doses of flagella. Above 1 ng, however, the response curve falls off. We have remarked before on some of the strange features of the response of rats to flagellin (Nossal et al., 1964a). The present survey has confirmed and extended the previous findings.



FIG. 8. Summary of results with monomeric flagellin. Note the 'flat' nature of the dose-response curves and the difficulty of drawing lines of best fit (see text). \triangle , Adjusted excess net secondary titres for groups receiving variable primary doses of flagellin and a constant secondary dose of 10 µg of flagella; •, adjusted excess net secondary titres for groups receiving the same dose of flagellin as primary and secondary stimulus; \times , primary response to flagellin.

Immunological Memory

SEX DIFFERENCES IN RESPONSIVENESS

Mean peak primary response 7S and 19S titres were calculated separately for males and females in each group. Small differences $(<1 \log_2)$ in titre were frequent between the sexes, sometimes favouring males and sometimes females. The only substantial difference was in the group receiving 100 µg of flagella, where the 19S peak titre was 2 log₂ higher in the females than in the males, the difference being statistically significant (0.05 > P > 0.01). However, we are reluctant to draw conclusions from this finding without further study.

DISCUSSION

This report compares the primary and secondary responses of rats to two flagellar antigens; the particulate flagella and the soluble protein flagellin. The first question asked was: does the secondary response represent merely a heightened version of the primary, with, therefore, similar kinetics of serum antibody rise? The answer was clear cut: the duration of excess antibody production was much shorter in the secondary than in the primary response. Next, conditions allowing demonstration of memory were investigated in detail, two quantitative measures of memory having been defined, the adjusted excess net secondary titre and the memory factor. The following were the main results.

(1) All primary doses of both antigens of 100 pg or greater induce a demonstrable state of memory, even though 100 pg and 1 ng rarely induce detectable primary antibody formation. However, to *evoke* this memory, the dose of the secondary injection needed to be equal to or greater than the first dose.

(2) The amount of memory induced by primary injections of flagella was dose-dependent over a wide range. The kinetic aspects resembled the early 19S primary response but not the 7S primary response.

(3) The 'excess' antibody in the secondary response was solely 19S with small antigen doses and 19S and 7S with larger doses. This was so even when the antigen used was soluble flagellin, which does not elicit detectable 19S antibody when given as a single injection.

There are two chief rival explanations for the phenomenon of specific secondary responsiveness. The first (Burnet, 1959) postulates the existence after primary immunization of an increased number of cells exhibiting reactivity with respect to the priming antigen. The second (Richter and Haurowitz, 1960) suggests that in primarily immunized animals, 'antigen-antibody complexes form immediately after re-injection of the antigen, which may act like particulate antigens or antigens given with an adjuvant'. Certain features of our studies with flagellin are consistent with the latter view, such as the fact that after secondary injection, flagellin produces a rapid 19S response, therein raising the possibility that a larger, more 'polymer-like' immunogen is produced by the interaction of monomeric flagellin and antibody. However, Richter and Haurowitz's postulate cannot possibly explain the bulk of the kinetic data presented here. In particular, the increasing amount of memory but almost constant amount of antibody produced by primary doses of 100 ng and above argue against their view. On the other hand, if the enhanced response is due to an increased number of reactive cells, it is clear that the detailed way in which this enlarged population responds to antigen is different from the way in which their ancestors had responded to the first injection. Two key differences are a rapid decline from peak antibody levels, and brisk appearance of both 19S and 7S antibody in the secondary response. Moreover, the lack of close correlation between primary response titre and

amount of memory suggests that production of 'memory cells' was not a direct and constant side-product of the cellular proliferative events leading to antibody formation, but rather a related but independent process induced in the lymphoid system by antigen.

It seems worthwhile to stress the fact that in our system, we are dealing with an antigen which is part of a pathogenic organism. It thus seems reasonable to seek teleological explanations for our results. If it could be shown that 19S and 7S antibodies served different purposes in combating an infection, then one requirement of an effective secondary response might well be to supply rapidly that component not present in the animal at the time of re-infection, namely 19S antibody. It has recently been shown (Humphrey and Dourmashkin, 1965) that it requires c. 700 molecules of 7S antibody but only one to two molecules of 19S antibody to lyse a red cell in the presence of excess complement. It is tempting to speculate (J. H. Humphrey, personal communication) that the defence of the host against particulate antigens such as bacteria is to produce a short burst of 19S antibody to allow the lysis of the invading organism followed by prolonged production of 7S antibody to neutralize soluble toxins. In any case, in the secondary response extremely high antibody levels, though useful for short periods, would be very wasteful if they persisted indefinitely. Perhaps some negative feed-back mechanism has been developed and is activated rapidly in the secondary response.

Some controversy has centred around the question of 19S memory. In two extensivelyinvestigated systems, namely the response of guinea-pigs to phage antigens (Uhr and Finkelstein, 1963) and of rabbits to poliomyelitis virus (Svehag and Mandel, 1964), small doses of antigen given as a primary injection caused only 19S antibody formation with no subsequent 7S phase. Apparently no memory results, as a similar dose given some weeks later leads to an almost identical response. In our system, using flagellar antigens in rats, small antigen doses (e.g. 10 ng) fail to elicit a detectable 19S response but lead to considerable 7S antibody formation and memory. Still smaller doses, e.g. 100 pg, lead to no detectable antibody formation but slight memory. The excess antibody formed in a subsequent secondary response is 19S in character, and though considerable individual variation was encountered, the levels of excess 19S antibody formed were sometimes quite high. In some ways, therefore, our secondary response resembled the primary response in the other two systems. As readily-detectable 'natural' antibody was frequently found against phage and polio antigens, but not against Salmonella adelaide flagella, it is possible that with the former antigens a first injection elicited a secondary response, the priming stimulus having been small doses of the antigens entering per via naturalis; whereas with our antigens, the first injection stimulated a true primary response. Our previous single cell studies on 19S and 7S antibody production (Nossal et al., 1964) showed that some individual plasmablasts and plasma cells synthesized only 19S antibody during their life-span and others switched over to 7S antibody production. It now appears likely that in our secondary responses, large antigen doses increased the proportion of cells undergoing such a switchover.

Impressive secondary responses could only be obtained when the second antigen dose was equal to, or preferably greater than, the primary dose. The simplest explanation of this seems to be that a secondary response can only occur when levels of antigen in phagocytic cells are raised (Ada *et al.*, 1965). At the time of our secondary injections, antigen levels in lymph nodes would still have been at about one-fifth of peak levels (Ada *et al.*, 1964a). Obviously, injection of one-tenth of the original dose could not raise these levels substantially. One puzzling feature is the failure of the antigen remaining in lymph nodes to induce a secondary response of its own accord. In an animal given, say, 10 µg of flagella as a first injection, much antigen is still present at 6 weeks. Obviously, in view of the excellent secondary responses which can be obtained with a further injection of 10 or 100 µg, there exist also many 'sensitized cells'. Why does the original antigen not provide a sufficient stimulus to fire the sensitized cells off into antibody production? One possibility is that the effectiveness of the antigen is blocked by an inhibitor. This may be antibody itself, as the work of Möller (1964) and Uhr and Baumann (1961) have suggested, or some more subtle factor. Re-injection of small amounts of antigen would not effectively alter the balance of power between antigen and inhibitor. However, increasing local concentrations would more and more effectively neutralize such inhibition, with results similar to those described above-the greater the disparity in amount between second and first dosage levels, the greater the size of the secondary response.

Finally, the practical object of our studies has been achieved. For future, critical work on the role of antigen in the induction of a secondary response, it seems desirable (a) to give a primary dose capable of inducing impressive, though not necessarily maximal, memory; and (b) to evoke a secondary response by an injection of an equal, or preferably somewhat larger, dose. For flagella, an optimal system seems to be 100 ng as a primary and 1 µg as a secondary dose. For flagellin, it is more difficult to establish optimal conditions in view of the 'flat' dose-response curves, but a similar dosage combination appears satisfactory. The next paper of this series will deal in detail with the localization, cellular distribution and fate of ¹²⁵I-labelled flagellar antigens in the secondary response.

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