Mechanism of Induction of Immunological Tolerance

IV. THE EFFECTS OF ULTRA-LOW DOSES OF FLAGELLIN*

G. R. Shellam and G. J. V. Nossal

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

Summary. Minute amounts of *Salmonella adelaide* flagellin were shown to be capable of inducing tolerance in newborn Wistar rats. With the range of doses, 10^{-9}–10^{-1} μg, the following results were obtained:

(1) Tolerance could be induced in two zones of dosage by daily injections of flagellin during the first 2 weeks of life. The low zone corresponded to a dose of 10^{-7} μg/g body weight and the high zone to 10^{-3} μg/g body weight. Doses between these evoked an immune response.

(2) Daily injections of flagellin for 6 weeks resulted in a shift in the dosage required for low zone tolerance from 10^{-7} μg/g body weight to 10^{-5} μg/g body weight.

(3) The events of low zone tolerance occurred at an antigen concentration, which, in an organ such as the spleen of a newborn rat, never exceeded about 10^{-14} M. The high zone of tolerance corresponded to an antigen concentration of about 10^{-10} M.

INTRODUCTION

Immunological tolerance induced by substantial amounts of antigen has long been an accepted phenomenon. However, the recent reports of Dresser (1962) and Mitchison (1964) that microgram doses of bovine γ-globulin and bovine serum albumin (BSA) respectively can induce tolerance in mice, have indicated that under certain circumstances very small amounts of antigen can be effective in tolerance induction. Indeed the study of Mitchison (1964) of the effect of sub-immunogenic doses of BSA in tolerance induction led to the concept of two zones of tolerance, corresponding to very small and to very large doses of antigen. Subsequently other antigens were found to evoke an identical pattern of response in mice, and the idea of a universal threshold for all antigens in the low zone of tolerance was put forward (Mitchison, 1967). However, there have been no reports of two zones of tolerance with antigens as immunogenic as *Salmonella* flagellin.

Prolonged treatment of rats from birth with microgram amounts of the soluble monomeric flagellin antigen of *Salmonella adelaide* can induce a state of complete tolerance (Nossal, Ada and Austin, 1965). It seemed pertinent, therefore, to investigate the response of rats to sub-immunogenic doses of this antigen in order to establish if the two zone effect could also be elicited. The design of the experiment was influenced by two factors, namely: (i) the inherent immunogenicity of the antigen (10^{-3} μg is immunogenic); and (ii) the very rapid clearance of this antigen from the circulation and lymphoid organs, even in newborn animals.

In the present study, we departed from the twice weekly injection schedules used for tolerance induction in previous papers (Nossal *et al.*, 1965; Nossal and Austin, 1966). Instead we instituted a daily injection regimen. The range of doses used was from 10^{-9} to 10^{-1} μg/g body weight of recipient animal. Challenges were of 10 μg of monomeric flagellin. Results are presented which demonstrate two zones of antigen dosage capable of inducing tolerance in this system. The low zone threshold has been found to require remarkably small doses of antigen.

MATERIALS AND METHODS

Animals

Newborn outbred Wistar albino rats of both sexes, aged less than 24 hours at their first injection, were used. Mothers were fed on 'Barastoc' dog cubes and tap water. When newborn litters were used for studies on the clearance of radioactively labelled antigen, mothers received tap water containing 4·5 g NaCl and 100 mg KI per litre, for about 1 week prior to parturition. For routine collection of serum, animals were bled from the tail under light ether anaesthesia.

Antigen

The soluble protein antigen monomeric flagellin, molecular weight about 38,000, was prepared from *Salmonella adelaide* SW 1338 by the method of Ada, Nossal, Pye and Abbot (1964b). Flagellin was always prepared from polymerized flagellin and used shortly afterwards. Special precautions were taken to minimize possible losses of antigen by adsorption on to glass at the very low dilutions. The diluent, normal saline, always contained some protein, either 0·1 per cent rat serum albumin or an equivalent amount of bovine serum albumin or 0·1 per cent normal rat serum. Preliminary experimentation had established that the nature of the protein in the diluent did not affect tolerance induction or immune responses. In addition, siliconized glassware was used. Antigen dilutions were made in standard flasks with microlitre pipettes down to 0·1 pg/ml. Injections were given intraperitoneally, or in very young animals intraperitoneally through the muscles of a hind leg, to prevent leakage of antigen. Antigen doses will be cited as micrograms (μg, 10^{-6} g), nanograms (ng, 10^{-9} g) and picograms (pg, 10^{-12} g).

Iodinated antigen

Flagellin was iodinated with ^{125}I by the chloramine-T oxidation method as modified by Ada, Nossal and Pye (1964a). Newborn rats were injected intravenously into the eye vein or intraperitoneally with microlitre amounts of [^{125}I] flagellin. Rats were killed at times which varied from 30 seconds to 3 days after injection, and the spleen and blood of each was removed. The spleens were placed in formalin solution immediately after removal, and both the spleen and blood were weighed. The radioactivity of all samples was determined at the end of the experiment by scintillation counting over a sodium iodide crystal.

Antibody titration technique

Serum samples were heated at 56° for $\frac{1}{2}$ hour and either titrated immediately or stored undiluted at −20°. Serum anti-H antibody levels were measured by a bacterial immobilization assay described by Ada *et al.* (1964b). Titres were expressed as the reciprocal of the dilution of serum giving a standard end point.

Computer analysis of results

All data were entered on IBM punched cards for statistical analysis by computer as described elsewhere (Jaroslow and Nossal, 1966). Serum antibody titrations were commenced at a serum dilution of 1:5. If this starting dilution contained no detectable antibody, the sample was assigned a titre of 1, which the computer, in calculating the geometric mean, coverted to $\log_{10} 1$, i.e. 0. In the tables which follow, we have used the titre value 0 to describe only those groups in which every single animal of the group had a titre of <5. On many occasions, the geometric mean calculated by the computer resulted in a titre value of less than 5. This, in fact, indicated that some animals in the group contained no detectable antibody, but that others had a titre of 5 or greater.

RESULTS

THE CLEARANCE OF FLAGELLIN IN NEWBORN RATS

The flagellin antigen is very rapidly removed from the circulation and organs of neonatal rats. The work of Mitchell and Nossal (1966) and, in particular, some of our own observations, indicate that the bulk of injected antigen is removed within 24 hours.

TABLE 1

CLEARANCE OF FLAGELLIN IN NEWBORN RATS

Time after injection	Blood		Spleen	
	i.v.	i.p.	i.v.	i.p.
30 seconds	170		22	
1 hour	53	53	177	206
4 hours	38	46	99	70
8 hours	39	41	37	66
16 hours	29	28	24	19
24 hours	22	18	20	15
90 hours	9	9	8	10

Results expressed as counts/sec/mg tissue. $0 \cdot 1$ μg of [^{125}I]flagellin with $1 \cdot 38 \times 10^5$ counts/sec was injected either intravenously (i.v.) or intraperitoneally (i.p.) into each rat.

In one experiment, groups of neonatal rats were injected either intravenously or intraperitoneally with $0 \cdot 1$ μg of [^{125}I]flagellin. Their blood and spleens were removed at various times later for assay of radioactivity. The results of this experiment are shown in Table 1. It can be seen that there is about an eight- to fourteen-fold decrease in the amount of antigen present in both the blood and the spleen during the first 24 hours by comparison with the peak amounts at 30 seconds and 1 hour, respectively.

Taking the spleen as an example of a lymphoid organ and considering it to weigh 15 mg in the newborn, then at 1 hour it has 1/50 of the injected antigen and only 1/500 by 24 hours. Obviously, then, to maintain even relatively stable antigen concentrations in lymphoid tissues, a daily injection protocol is necessary.

THE EFFECT OF AGE ON TOLERANCE INDUCTION

The basic model of tolerance induction in the flagellin system, described previously (Nossal *et al.*, 1965) consists of the injection of rats with 10 μg of flagellin twice weekly,

beginning at birth. It has been subsequently observed that 1-week-old animals injected with 10 μg twice weekly also developed full tolerance (Table 2). However, if 2-week-old animals are put on this multiple injection schedule, they form some antibody, though not developing very high titres. Clearly then, the first few weeks of life are crucial ones for the induction of full tolerance in this system.

With this knowledge in mind, the following experiment was designed to investigate the effect of very small amounts of antigen given daily during the first 2 weeks of life, and to determine the smallest dose capable of inducing tolerance in the animals.

TABLE 2

THE EFFECT OF AGE ON TOLERANCE INDUCTION

Age at first injection	No. of animals	Mean antibody titre					
		5 weeks	8 weeks	12 weeks pre-challenge	1 week post-challenge	2 weeks post-challenge	6 weeks post-challenge
<24 hours	8	1	1	0	1	4	9
1 week	9	0	1	1	2	6	ND
2 weeks	10	25	166	12	17	17	ND
Controls (12 weeks)	9			0	1	24	105

Animals were given a course of 10 μg flagellin twice weekly until 10 weeks of age, beginning at birth, 1 or 2 weeks of age. Ten micrograms flagellin challenge was given 2 weeks after the last injection. Controls received the challenge injection only.

EXPERIMENTAL PROCEDURE

Daily injections of the range of doses 10^{-15}–10^{-7} g of flagellin/g body weight were given to rats of both sexes, beginning on the day of birth, and continuing for 2 weeks. Usually two or three litters were used for each dosage. Control rats received diluent only (0·01 ml/g body weight) during this period. All rats were given twice weekly injections of 10 μg of flagellin, beginning the day after daily injections ceased, and continuing until the animals were 10 weeks of age. Then followed a rest period of 2 weeks. At 12 weeks of age, rats received a challenge of 10 μg of flagellin. Then followed a further rest of 6 weeks, and at 18 weeks of age a final injection of 10 μg of antigen was given. Serum samples were taken when the rats were aged 6, 8, 10, 12, 13, 18, 19 and 24 weeks, and were titrated for antibody content.

INDUCTION OF TOLERANCE IN TWO ZONES OF DOSAGE

The pooled results of this study are presented in Table 3 and Figs. 1–3. Attention must be first drawn to the fact that the tolerance-maintaining regimen instituted at age 2 weeks and continued until age 10 weeks (controls, Table 3) in fact produced a substantial degree of partial tolerance in its own right. Thus, antibody titres in the control group reached a peak at age 8 weeks and subsequently declined, essentially failing to respond to challenge at age 12 and 18 weeks. Accordingly, the chief feature of interest in this experiment relates to the immune status of the various groups at age 8 weeks. At that time, rats given diluent only over the first 2 weeks had made a substantial amount of antibody to the twice weekly inoculations of 10 μg of flagellin, given between 2 and 8 weeks.

TABLE 3

THE EFFECT OF DAILY INJECTIONS OF FLAGELLIN FOR 2 WEEKS*

Antigen dose	No. of animals	Mean antibody titre								†P
		6 weeks	8 weeks	10 weeks	12 weeks (pre-challenge)	1 week post first challenge	6 weeks post first challenge	1 week post second challenge	6 weeks post second challenge	
200 ng	6	1	0	0	0	0	0	0	0	$0.001 > P > 0.0$
20 ng	4	0	0	0	0	0	0	0	0	$0.001 > P > 0$
2 ng	7	2	3	2	1	0	0	0	0	$0.001 > P > 0$
100 pg	15	66	28	12	14	8	6	480	19	$1.0 > P > 0.1$
10 pg	32	199	149	90	40	30	6	10	26	$1.0 > P > 0.1$
1 pg	26	19	17	7	9	8	1	10	5	$0.05 > P > 0.02$
0·1 pg	27	7	3	2	1	1	1	9	1	$0.01 > P > 0.001$
10^{-2} pg	17	28	11	4	3	7	1	8	3	$0.01 > P > 0.001$
10^{-3} pg	21	41	27	15	14	6	1	28	11	$0.01 > P > 0.001$
Controls	10	91	116	72	62	33	6	16	10	$1.0 > P > 0.1$

* Daily injections of the above doses were given to rats for the first 2 weeks of life, followed by twice weekly injections of 10 μg of flagellin until the animals were 10 weeks old. Challenge was with 10 μg flagellin at 12 and 18 weeks of age. Controls received diluent only for the first 2 weeks of life and then identical treatment to the other groups.
† P values for comparison of the 8-week control titre and the corresponding titre of each other group.

The striking feature of the results is the occurrence of two zones of tolerance, as judged by the titres at 8 weeks. Complete tolerance is seen with doses of 2 ng/g body weight and above. A very substantial degree of partial tolerance ($P<0.001$) is seen following daily injections of 0·1 pg in the first 2 weeks. These two zones of tolerance are separated by an 'immunity hump', as in Mitchison's (1964) model. In our system, however, the mean amount of antibody made by rats injected with 10 pg/g body weight daily, does not significantly exceed the mean titre of control animals (Table 3). However, some individual titres in the 10-pg group were markedly elevated, the highest titre being ten-fold higher
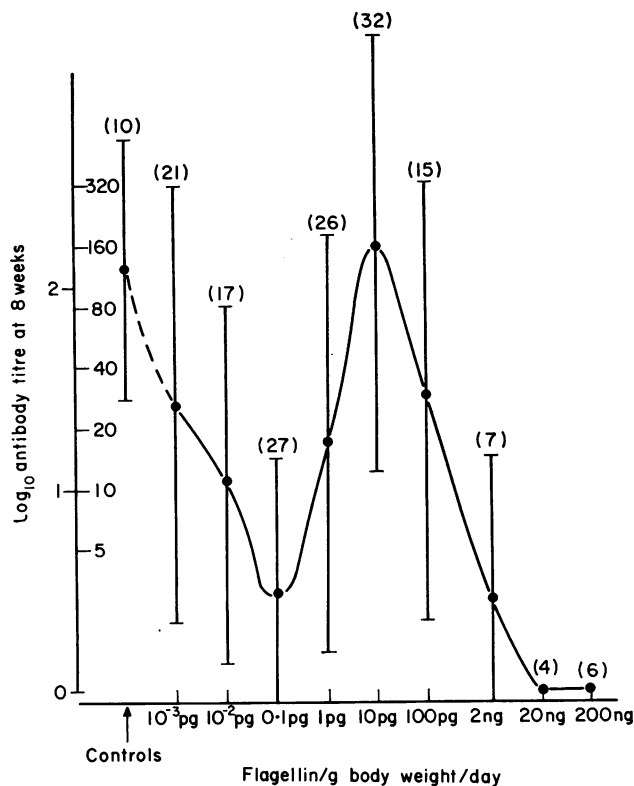

FIG. 1. Induction of tolerance by daily injections of various doses of flagellin from birth for 2 weeks, followed by 10 µg flagellin twice weekly until 10 weeks of age. The number of animals in each group is listed in parentheses.

than the highest titre of the control group. This individual variability in response, reflected by the large standard deviations (Fig. 1, vertical bars), is a characteristic feature of our work with flagellin (Nossal, et al., 1965).

In Fig. 2, the response at 8 weeks is expressed as a percentage of the control response, and in Fig. 3, the percentages of animals in each group having complete tolerance (titres <5) are given. It is striking to note that the extremely small dose of 0·01 pg (10^{-14} g)/g body weight still induces statistically significant low zone tolerance ($0.05>P>0.02$). Even 10^{-15} g may have had some effect on some animals (Fig. 3), although the means did not differ significantly from those of the controls ($1>P>0.1$).

The high zone of tolerance induced with doses above 1 ng/g body weight daily, is also

of some interest. Previously, because of the extremely small amounts of antigen needed, we had regarded tolerance achieved by twice weekly injections of a total of 1 μg/rat as low zone tolerance. The present study: (a) has shown that this tolerance was in fact an
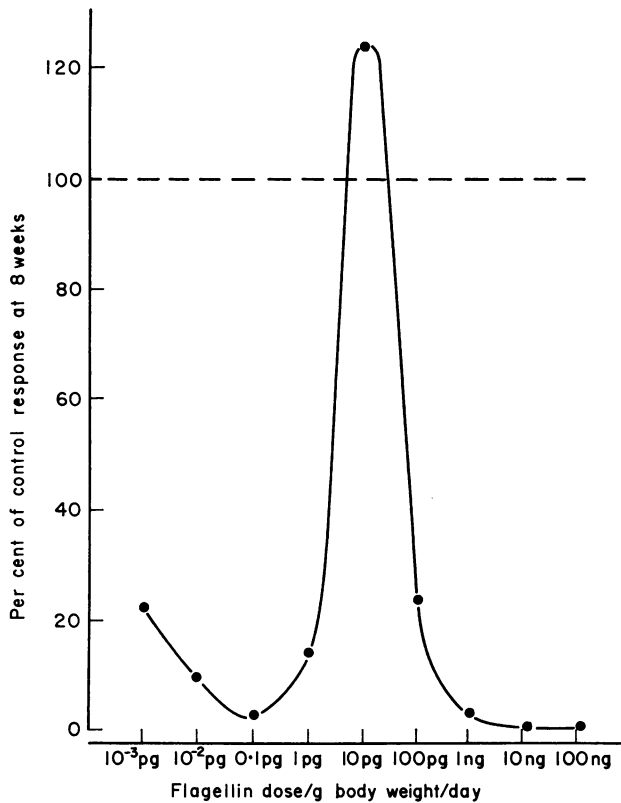

FIG. 2. Mean serum antibody titres at 8 weeks expressed as a percentage of the control titre.

TABLE 4

THE EFFECT OF DAILY INJECTIONS OF ANTIGEN
ON TOLERANCE INDUCTION

Antigen dose*	Mean antibody titre at 8 weeks
0·1 pg/g body weight daily for 10 days	95
0·1 pg/g body weight daily for 14 days	3

* Both groups received 10 μg flagellin twice weekly from day 15 onwards.

example of high zone tolerance; and (b) has defined an approximately 100-fold lower threshold through the use of daily rather than twice-weekly injections.

The need for daily injections is emphasized by an accidental error. One group of rats receiving 0·1 pg daily, missed the last four injections prior to the beginning of the accompanying twice-weekly injection course (Table 4). Their mean titre at 8 weeks of age

contrasts with the titre of 3 of the properly injected group, suggesting that even in 4 days enough cells can escape the events of tolerance to give a significant immune response to the subsequent multiple injections of antigen.
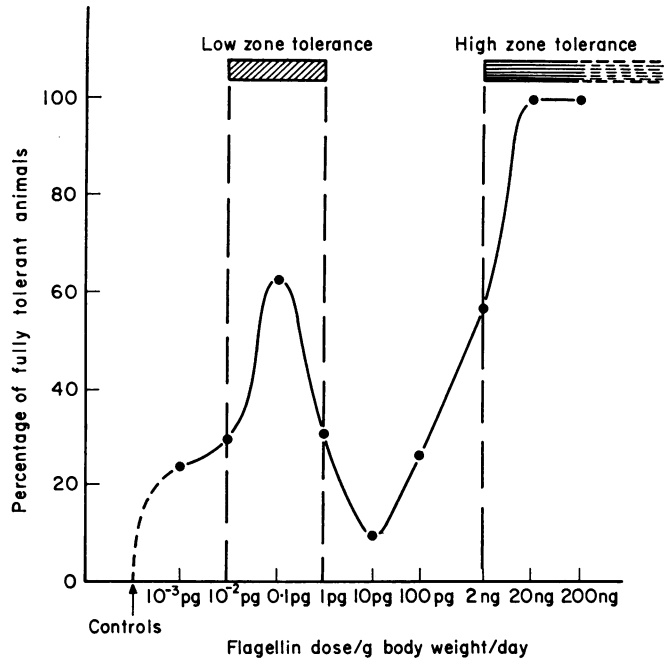

FIG. 3. The percentage of fully tolerant animals at 8 weeks of age.

INDUCTION OF TOLERANCE BY DAILY INJECTIONS OF FLAGELLIN FOR 6 WEEKS

The above experiment was designed to investigate the question of tolerance induction during the first 2 weeks of life, and it employed a tolerance maintaining schedule of 10 μg flagellin twice weekly from the 2-week timepoint onwards. Another experiment was performed to determine the effect of a 6-week barrage of daily injections of the same range of doses as before, in an environment where antigen trapping and localization mechanisms were constantly maturing (Mitchell and Nossal, 1966), and where a mature antibody response could be expected to challenges with flagellin given at the end of the injection course. Rats were given such daily injections for the first 6 weeks of life and were challenged with 10 μg flagellin at 6 weeks and 10 weeks of age. Control animals received daily injections of diluent only and were challenged at the same time as the test animals. The range of doses studied was 10^{-9}–10^{-4} μg/g body weight. The results are shown in Table 5.

An interesting fact that emerges from this study is the shift in the dose needed for low zone tolerance. In fact, a daily dose of 10 pg is needed, an amount of antigen which if given for 2 weeks, as in the first experiment, gives rise to an 'immunity hump'. The highest dose used, 100 pg/g gave antibody formation in some animals and tolerance in others. This phase of the study was not continued into the high zone tolerance antigen range.

As regards the lowest antigen doses used in the study, it should be noted that extreme individual variation was encountered and that thus the geometric means of the 10^{-3} and the 10^{-2} pg groups, though apparently so different, did not differ to a statistically significant degree from each other. The next group, 10^{-1} pg, may actually have exhibited a latent state of priming which only manifested itself after two challenges. However, the differences between it and the controls were also not statistically significant.

TABLE 5

THE EFFECT OF DAILY INJECTIONS OF FLAGELLIN FOR 6 WEEKS

Antigen dose (pg)	No. of animals	Mean titre pre-challenge	Mean titre 4 weeks post first challenge	Mean titre 1 week post second challenge	Coefficient of variation*	P†
10^{-3}	7	0	14	6,385	49·3	$1.0 > P > 0.1$
10^{-2}	5	0	3	172	98·3	$1.0 > P > 0.1$
10^{-1}	14	0	6	15,814	27·3	$1.0 > P > 0.1$
1	14	0	17	1,697	40·1	$1.0 > P > 0.1$
10	15	0	0	102	58·9	$0.01 > P > 0.001$
100	13	13	21	118	80·5	$0.05 > P > 0.02$
Controls	6	0	10	5,814	22·0	

Rats were given the above range of doses daily for 6 weeks followed by an immediate challenge of 10 μg flagellin and another 4 weeks later. Controls received diluent only for 6 weeks, with the same challenges.

* Coefficient of variation = (SD × 100 ÷ mean), relating to 1 week post second challenge titres.
† P values for comparison of the 1 week post second challenge titre of the controls and the corresponding titre of each group.

The results of this study, which used a somewhat more conventional tolerance-inducing regimen, confirm the presence of a low zone of tolerance and show that this is a fundamental phenomenon which was not merely dependent on the particular experimental protocol adopted in the first experiment. It should be noted that every one of fifteen rats in the 10-pg group showed complete tolerance to a first challenge, and titres following second challenge were far below control values in twelve of fifteen animals in this group.

MOLAR CONCENTRATION OF ANTIGEN IN SPLEEN DURING TOLERANCE INDUCTION

We have made an attempt to estimate the probable molar concentrations of antigen in a typical lymphoid organ such as the spleen during tolerance induction in the newborn period. This calculation rests on the untested assumption that the distribution of 100 ng

TABLE 6

ANTIGEN CONCENTRATIONS INDUCING TWO ZONES OF TOLERANCE DURING THE FIRST 2 WEEKS OF LIFE

	Low zone	Immunity hump	High zone
Antigen dose (g)*	5×10^{-13}	5×10^{-11}	5×10^{-9}
Concentration of antigen in the spleen 1 hour after injection (M)	3×10^{-14}	3×10^{-12}	3×10^{-10}
Concentration of antigen in the spleen 24 hours after injection (M)	2×10^{-15}	2×10^{-13}	2×10^{-11}
Approximate number of antigen molecules in the spleen 1 hour after injection	2×10^5	2×10^7	2×10^9

* Each dose was given on a per gram of body weight basis, and the newborn rat weighs approximately 5 g.

E*

of iodinated antigen after intraperitoneal injection (Table 1) mirrors accurately the distribution of the much smaller amounts of uniodinated antigen used in the study. The results (Table 6) are given only over the first 24 hours. However, we know that the antigen levels would only creep up very slowly over the 2-week period under consideration. Table 6 shows that the trough of low zone tolerance is induced when the median antigen concentration in the spleen is about 10^{-14} M.

DISCUSSION

The chief findings of the study were:

(a) Two zones of tolerance to flagellin can be induced in newborn rats by the daily injection of surprisingly small doses for 14 days. A dose of 0·1 pg/g body weight results in low zone tolerance and $\geqslant 2$ ng/g body weight causes high zone tolerance. An immune response is evoked by doses between these and it peaks at a dose of 10 pg/g body weight.

(b) Continual injections of these doses for a 6-week period result in a shift of the low zone of tolerance to a dose of 10 pg/g body weight, i.e. a 100-fold higher dose than observed in the above study.

The concept of two zones of tolerance, introduced by Mitchison (1964), has been strengthened by these results. Furthermore, it is now apparent that both strong and weak antigens have the propensity for low zone induction. But whilst flagellin in newborn rats gives rise to the same general pattern of response as BSA in adult mice, certain features vary greatly from Mitchison's model.

Most importantly, flagellin is effective in much lower doses in the induction of both tolerance and immunizing, than BSA. In fact, there is a concentration of only 10^{-14} M in the spleen and extracellular fluids when the sort of dose needed for low zone tolerance is given, contrasting strikingly with the 10^{-8} M concentration of BSA for low zone tolerance in adult mice. This negates Mitchison's (1967) idea of a universal threshold for all antigens in low zone induction and suggests that flagellin is about 10^6 times better at tolerance induction than BSA. Moreover, the exquisite sensitivity of the tolerance mechanism is strongly emphasized by the success of these tiny doses. We do not know the actual site at which lymphoid cells are rendered tolerant. This could be within the peripheral lymphoid organs, the thymus, the bone marrow or the circulation. In fact, the antigen concentrations present in these various sites would have differed very little over the first 2 weeks of life. Table 6 shows that about 10^5 molecules of flagellin is near the maximum antigen concentration achieved in the spleen. If the spleen is a site of tolerance induction, the ratio of number of antigen molecules : nucleated cells is less than 1:100. In previous work (Mitchell and Nossal, 1966) we have shown that there are no obvious sites of antigen accumulation or concentration in lymphoid organs of newborns. In all, the results suggest that it is unlikely that any cell ever encounters more than one antigen molecule at any one time during low zone tolerance induction. Although no mechanism of tolerance can be deduced from these results, it is difficult to see how antigen in such low concentration could get to some controlling site in a cell, or could cause the inactivation of a gene. Some mechanism of union of a single antigen molecule with a single molecule of natural antibody on the surface of cells, with consequent destruction, seems more plausible. In any case, older notions of 'paralysis' of cells by overwhelming antigen concentrations within them must clearly be discarded as incorrect.

Another point arising from this study is the need for the continued presence of antigen during tolerance induction. By the simple expedient of giving injections daily instead of twice-weekly, we have lowered the threshold for high zone tolerance by a factor of 100. In low zone tolerance, omission of antigen for only 4 days allowed significant numbers of cells to escape tolerance induction and respond to subsequent challenge. Presumably, the need for repeated injections reflects the short half life of flagellin in the circulation and organs of the rat (Table 1).

The shift in required antigen concentrations between the two main experiments is of interest. When tolerance was induced by daily injections for 2 weeks followed by a strong tolerance-maintaining regimen, the trough of low zone tolerance corresponded to 10^{-1} pg/g body weight, but when daily injections of minute amounts were continued for 6 weeks, the trough of low zone tolerance corresponded to 10 pg/g body weight. This may reflect an increasing maturity of the antigen-capturing system of the host. Both the level of opsonic factors and the maturity of the reticulo-endothelial system probably increase substantially between 2 and 6 weeks. In the face of increased removal of antigen over this period, more antigen may have to be given daily to achieve the same tolerance-inducing effect.

Mitchison's finding that the threshold for tolerance induction can be less than that for immunity induction has been confirmed. Tolerance must now be regarded as perhaps the most basic of all immunologic phenomena—the most likely result of an interaction between an antigen-reactive cell and a molecule of antigen. Immunity requires the presence of more antigen, either because it has a higher threshold, or because it requires antigen processing by macrophages or localization of antigen on the dendritic processes of reticular cells, before the cellular proliferative events can begin. Any more detailed discussion of the cellular basis of low zone tolerance is premature. Current efforts in our laboratory are directed towards the question of adult tolerance in this system, and results will be reported in later experiments in this series.

ACKNOWLEDGMENTS

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