*LOW ZONE TOLERANCE TO BACTERIAL FLAGELLIN IN ADULT RATS: A POSSIBLE ROLE FOR ANTIGEN LOCALIZED IN LYMPHOID FOLLICLES**

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The flagella of *Salmonella* organisms are composed of smaller protein units—flagellin (mol wt about 40,000)—which can be readily polymerized to form particles (polymer) of similar structure to the original flagellar particle. Each form of the protein, when injected in submicrogram doses in saline into adult rats, is immunogenic, though injection of the particulate forms causes quicker and greater antibody responses.[1] Injection into adult rats of any of these forms of the antigen over a wide range of doses fails to cause demonstrable tolerance. Injection of flagellin into rats from birth (200 μg injected during ten weeks) results in tolerance to flagellin.[2] The flagellin molecule contains three methionine residues; upon treatment with cyanogen bromide, we obtain four main polypeptides, the largest of which is called fragment A (mol wt 18,000) and possesses all the demonstrable antigenic activity of the parent molecule.[3] It was recently shown that in adult rats tolerance to flagellin and to the polymer could be achieved by the daily injection of 100 μg of the complete CNBr digest of flagellin for four weeks[3] or of the isolated fragment A (Parish and Ada, in preparation). Following the work of Mitchison,[4] who showed that in adult mice tolerance to bovine serum albumin (BSA) could be obtained at high (5 mg) and at low (10–40 μg) dose ranges, we performed similar experiments with the CNBr digest of flagellin and with the isolated fragment A.

Demonstration of Low Zone of Tolerance.—Six- to seven-week-old Wistar rats (randomly bred, six to nine rats per group) were injected intraperitoneally (i.p.) daily for four weeks with amounts of the CNBr digest of flagellin varying in tenfold dilution steps from 100 μg to 10^{-3} picogram (pg). Figure 1 shows the antibody titres in the rats at the end of this four-week period. The rats were then injected in the hind footpads with 100 μg flagellin in Freund's complete adjuvant (FCA) and the titres compared with those of a control group injected only with flagellin in FCA. Antibody titres two weeks after the challenge injection showed two zones of tolerance separated by a region where immunity occurred (Fig. 2). The maximum low zone of tolerance was at the level of 10^{-1} pg of the digest. In a second experiment, adult rats were injected daily with fragment A rather than with the whole digest. A low zone of tolerance was observed when 10^{-1} pg and 1 pg were injected daily. In a third experiment, adult rats (20 per group) were injected daily with 10^{-1} pg of the CNBr digest of flagellin for two, three, or four weeks and then injected, together with a control group of 20 rats, with 100 μg of flagellin in saline. Rats injected for four weeks showed the greatest degree of tolerance (Fig. 3). At the two-week period after challenge, 8 of the 20 rats in this group had no detectable antibody titre, whereas all the rats in the control group had antibody titres. The lowered antibody re-

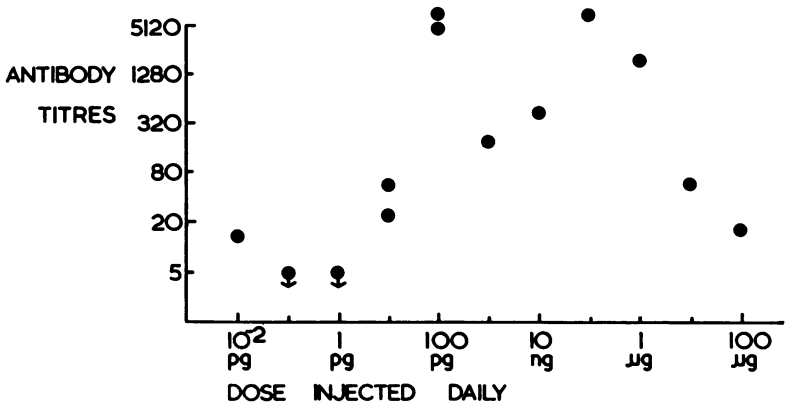

FIG. 1.—Antibody titres of Wistar rats (seven or eight per group) injected intraperitoneally daily for 28 days with varying amounts of the CNBr digest of flagellin. Antibody titres were measured by the immobilization technique.[1]

sponse is statistically significant (Fig. 3). This tolerance-inducing dose (10^{-1} pg per day) is lower than that reported by Shellam and Nossal[5] for a low zone of tolerance with flagellin in neonatal rats (10^{-1} pg/gm body wt) and much less than the figure for BSA in mice (10–40 µg).[4]

In any discussion of the possible mode of action of the injected material, it is desirable to know the fate of the injected antigen. It is a common experience that foreign soluble substances injected intravenously or intraperitoneally into animals become distributed widely around the body, with only a small fraction of the injected material being recovered in lymphoid organs. Three different

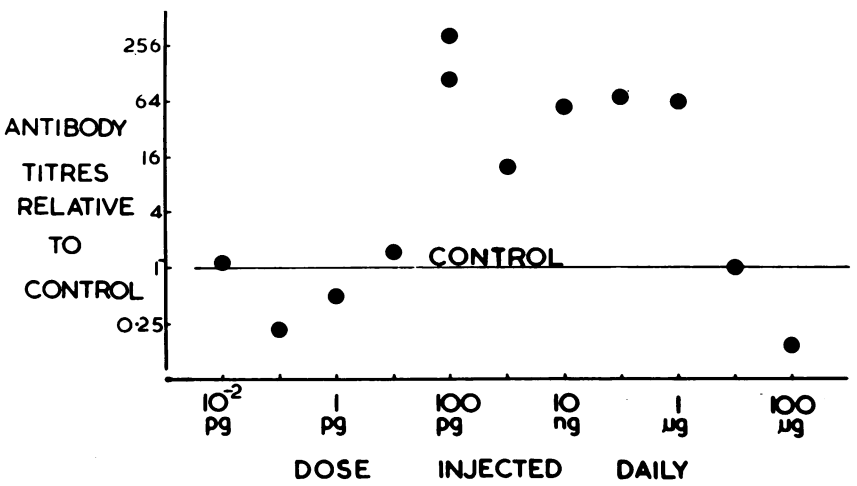

FIG. 2.—Antibody titres of rats (seven or eight per group) injected daily for 28 days with varying amounts of the CNBr digest of flagellin (Fig. 1) and then injected with 100 µg of flagellin in FCA. Antibody titres were estimated 2 weeks after this injection and in the figure are expressed relative to the titres of rats injected only with flagellin in FCA.

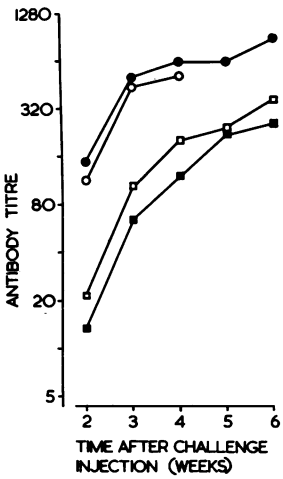

FIG. 3.—Antibody titres of rats (20 per group, 6–7 weeks old) injected daily for 14 (O—O), 21 (□—□), or 28 (■—■) days with 10^{-1} pg of the CNBr digest of flagellin and then, together with a control group of 20 rats (●—●), with 100 μg of flagellin in saline. The results of control (●) and 4-week injected (■) groups at the 2-, 3-, and 4-week time points were analyzed with nonparametric statistics (the Rank Test). In each case, the null hypothesis that the samples came from the same population was rejected ($p < 0.01$).

experiments were done with fragment A. Rats were injected intravenously with 10 μg of I^{125}-labeled[6] fragment A; the half life in the blood was found to be less than one hour. In another experiment, rats were injected intraperitoneally with I^{125}-labeled fragment A in doses ranging from 100 μg to 100 ng. After 24 hours, less than 0.1 per cent in each case was recovered in the spleen and other lymphoid tissues draining the peritoneal cavity. During the 28-day course of daily injections (10^{-1} pg of the CNBr digest of flagellin) in the tolerance experiments, the equivalent of $<10^8$ molecules of fragment A were injected. These experiments suggest that in the tolerance experiments substantially less than the total number of molecules of fragment A injected intraperitoneally may have reached the lymphoid system.

What happened to antigen which did reach the lymphoid system? Investigation of the fate of antigen in lymphoid tissues has been done by radioautographic examination of sections of lymph nodes, such as popliteal nodes, which drain the hind footpads of animals. Again, though only a small amount of the injected material is found in such nodes at any particular time after injection, the general pattern of distribution within a particular node has also been found in other nodes and in the spleen.[7, 8] I^{125}-labeled fragment A was injected into the hind footpads of Wistar rats. Compared with an earlier experiment that used I^{125}-labeled flagellin,[9] both antigens drained from the footpads at similar rates, but less fragment A than flagellin was recovered in the popliteal nodes (Fig. 4). Figure 5, a radioautograph of the popliteal node at 16 hours after injection, shows an absence of radioactivity over most of the cells, a relatively poor uptake of the antigen into medullary macrophages, and a very strong localization of the label in the lymphoid follicles. A comparison of these results with those obtained earlier with flagellin and polymerized flagellin[10, 11] indicates that a major difference is in the relatively poor uptake of fragment A into the medullary macrophages. Early localization of each antigen in the follicles is due to natural antibody.[12, 13]

Discussion.—The immune response which occurred after the injection into Wistar rats of the CNBr digest of flagellin over a wide range of doses—two zones

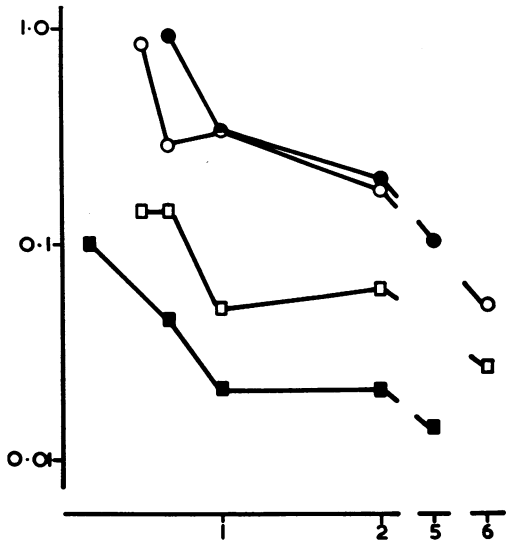

FIG. 4.—Radioactivity in the feet and popliteal lymph nodes of rats injected into the footpads with I^{125}-labeled flagellin or fragment A.

Ordinate: Percentage of total radioactivity injected ($\log_{10}$).

Abscissa: Time after injection (days).

Flagellin in footpads (O), in nodes (□). Fragment A in footpads (●), in nodes (■). Values for flagellin taken from ref. 9.

of tolerance separated by a region of immunity—was comparable in general outline to that observed for BSA in mice by Mitchison, except for the doses of antigen used. In particular, a low zone of tolerance to BSA was achieved in mice at levels of 10 μg BSA per injection but to flagellin in rats of 10^{-1} pg of fragment A per injection—a difference of one hundred million times or even greater if expressed per gram of body weight. How can this be explained?

The available evidence[14] indicates that tolerance is the lack of production of specific antibody to a particular antigen because of the inactivation of precursor lymphocytes. Dresser and Mitchison[14] propose that tolerance is the result of a direct reaction between lymphocytes and antigen. For tolerance to be demonstrated, the antigen, or some product or messenger derived from it, must react with a high proportion of specific lymphocytes (and thus potentially with a similar proportion of all circulating lymphocytes). If, in the present experiments, the number of molecules of fragment A postulated to reach the lymphoid system (say $<10^7$) was present in free solution, the probability of their interaction with a significant proportion of lymphocytes is very small. Thus, it seems necessary to invoke some amplification mechanism. Two possibilities are: (1) Antigen reacting with a few cells may generate many "messengers" which induce tolerance. Though this possibility is not excluded, lack of evidence precludes any useful discussion. (2) Antigen, the reagent in "shorter supply," may be concentrated in particular areas so that circulating lymphocytes may over a period of time come into contact with the antigen. The demonstration that fragment A may localize strongly in lymphoid follicles—a method of extracellular localization of antigen,[15–18] mediated by natural[13] or specific[9, 18–20] antibody—provides the justification for discussing this second possibility.

Hypothesis.—Mitchison[4] and others have shown that antibody production and tolerance can occur simultaneously after the injection of an antigen. If antigen in lymphoid follicles can induce immunological reactions, a simple explanation of

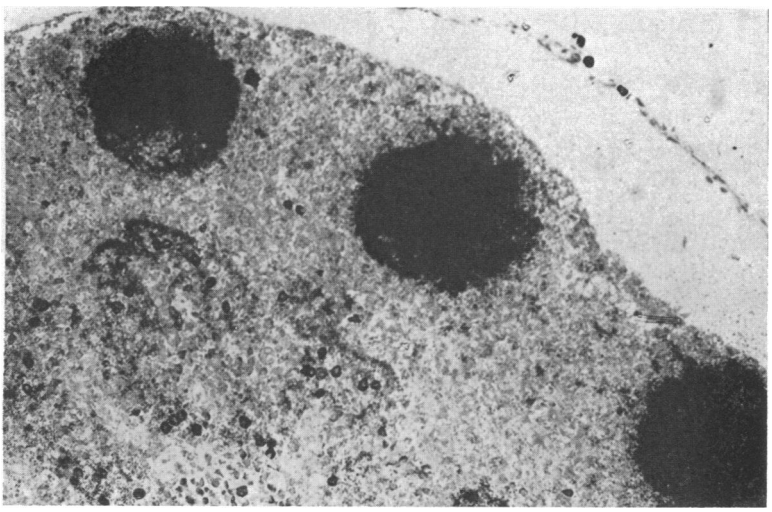

FIG. 5.—Autoradiograph of a section of the popliteal lymph node of a rat injected 16 hr previously with 10 μg of fragment A, labeled with I^{125}. Note the strong localization of the isotope in the lymphoid follicles relative to the medullary macrophages.

the present findings would be that circulating lymphocytes reacting in the follicles with very few or very many molecules of antigen were rendered tolerant; those reacting with intermediate numbers of antigen molecules were induced to differentiate and make antibody (Burnet, personal communication). Such a concept does not account for many observations (such as those in the work of Fishman and Adler[21] and many others) which implicate macrophage-antigen interaction in antibody induction or for the demonstration that antibody production occurs after localization of antigen in the medullary macrophages of lymph nodes with follicular localization of antigen occurring either not at all[22] or only *after* antibody is produced.[20] It has also been proposed that antigen localized in lymphoid follicles may be the method of preference for triggering primed cells.[22]

To account for these observations, it is suggested that antigen in medullary cells is concerned in the induction of antibody formation and of primed cells; antigen in lymphoid follicles may trigger primed cells or cause tolerance. Circulating antigen may induce tolerance but rather inefficiently.[20] Feedback inhibition of antibody formation by antibody[23] might be mediated by antigen caused to localize in lymphoid follicles. For antigens rapidly phagocytosed, the immune status of an animal would be a reflection of the relative and total amount of antigen over a period of time in these two different areas of lymphoid tissues. At present, such an hypothesis serves as a guide to the planning of further experiments; further discussion now is unwarranted.

Summary.—Partial tolerance in adult rats to bacterial flagellin, a potent immunogen, has been obtained by the daily injection for 28 days of a partially degraded flagellin preparation, equivalent in amount to a total of 10^8 molecules of flagellin. Of this amount, only a small portion may have reached the lymphoid

system. This finding places severe restraint on the possible ways in which antigen can be postulated to act to induce tolerance and suggests the necessity for some amplification mechanism. Radioautographs of lymph nodes from rats injected with larger amounts of I^{125}-labeled fragment A, the serologically active portion of the flagellin digest, showed strong localization of this substance in lymphoid follicles relative to the uptake in the medullary macrophages of the nodes. It is proposed that in the tolerance experiments, a portion of the injected antigen also localized in the follicles of the lymphoid tissue and that reaction of circulating lymphocytes with this antigen resulted in tolerance. However, the possibility that other amplification mechanisms may be involved is not discounted.

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