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MEASUREMENT OF ANTIBODY-PRODUCING CAPACITY IN MAN

I. THE NORMAL RESPONSE TO FLAGELLIN FROM *SALMONELLA ADELAIDE**

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SUMMARY

The immune response to a single subcutaneous injection of 5 μg of flagellin from *Salmonella adelaide* was examined in 108 human subjects. Titrations were performed by tanned cell haemagglutination before and 1, 2, 6 and 10 weeks after injection. The class of antibody was assessed by mercaptoethanol treatment of serum. Antibody which was entirely IgM was present in 86% of sera before immunization: this is referred to as 'natural' antibody. After injection of flagellin the response was predominantly IgM, but both IgM and IgG antibody reached peak titres at 2 weeks and both persisted throughout the response. By contrast after a second injection of flagellin the response was almost entirely IgG antibody.

The mean titres of total antibody were significantly higher in females than males, and in healthy subjects as compared with those attending hospital for miscellaneous illnesses. There was no significant quantitative difference in the response of subjects grouped according to their titre of 'natural' antibody, but titres fell more rapidly in the group with a low titre of 'natural' antibody. Mean titres of 'natural' antibody were significantly lower in aged than younger subjects, but there was no significant difference in peak titres after immunization. Immunization with flagellin should have considerable potential as a standard test of 'antibody-producing capacity' in man, and be applicable to the investigation of immune deficiency diseases and the effects of immunosuppressive agents.

INTRODUCTION

There are no reliable quantitative indices of the 'functional capacity' of the immunological system in man. The occurrence of infections, the levels of immunoglobulins, the elicitation of cutaneous delayed hypersensitivity reactions, and the antibody response to various vaccine antigens have all been used, but standard routine methods have not been developed.

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This paper describes an attempt to use antibody production to flagellin as one index of the functional capacity of the immune system, and describes the response in subjects considered to have a normal antibody-producing system.

Flagellin from *Salmonella adelaide* was chosen as the test antigen for the following reasons: (i) it can be prepared as a pure protein; (ii) it has been well characterized biochemically and antigenically; (iii) strains of Salmonellae sharing flagellar (f, g) antigens with *S. adelaide* are rarely isolated in the State of Victoria (Taplin, 1968 personal communication) and hence it should stimulate a primary response; (iv) the simple and reliable method of tanned cell haemagglutination (TCH) is available for titration of antibodies and provides a system in which treatment of serum with 2-mercaptoethanol (ME) partly identifies the class of antibody produced; and (v) the antibody response in rats has been well characterized and provides a comparison for the human response (Nossal, Ada & Austin, 1964).

METHODS AND SUBJECTS

Preparation of antigen

Monomeric flagellin was prepared from *Salmonella adelaide*, strain SW 1338. A single batch of flagellin was used throughout, and was stored at −20°C. It was prepared as described by Ada *et al.* (1964) using fresh, sterile reagents and keeping the flagella in the presence of 1:10,000 merthiolate. After preparation, the solution was passed through a sterile Seitz filter (No. 9 pad). The final protein concentration of the solution was 5·5 mg/ml.

Spontaneous polymerization takes place when monomeric flagellin is stored at −20°C; hence depolymerized antigen was prepared shortly before injection from a stock diluted with 0·15 M saline to 500 μg/ml, i.e. ten times the required concentration. Under sterile conditions, 0·05 volume of 1 N hydrochloric acid was added, the mixture was allowed to stand for 20 min, and was neutralized with an equal volume of 1 N sodium hydroxide. The solution was diluted to 50 μg/ml in phosphate-buffered saline, pH 7·0.

Immunization

Five micrograms of flagellin in 0·1 ml was injected subcutaneously into the forearm. Samples of blood were taken before injection and at 1, 2, 6 and 10 weeks after injection. 'One week' was regarded as 6–8 days after injection, '2 weeks' as 13–21 days, '6 weeks' as 35–49 days and '10 weeks' as 63–77 days. Preliminary information obtained from weekly testing had indicated that within these periods antibody titres altered little.

Measurement of antibody

Routine titrations of antibody in human serum were done by TCH (Wistar, 1968). A 4% suspension of washed sheep red cells, 7–14 days old, was treated with an equal volume of 1:25,000 tannic acid in veronal saline buffer, pH 7·3 (VSB), for 30 min. The cells were again washed and sensitized with polymerized flagellin from *S. adelaide*. The sensitized cells were used as a 2% suspension in VSB containing 0·05% bovine serum albumin (BSA). The test sera were inactivated at 56°C for 30 min, and absorbed with an equal volume of packed sheep red cells before titration. Doubling dilutions of serum in VSB containing 0·05% BSA were carried out from initial dilutions of 1:5, 1:160 or 1:1,000. The titration volume was 0·25 ml, and 0·1 ml of the 2% suspension of sensitized cells was added to each cup. Titrations were

allowed to settle for at least 2 hr before reading the end point. Each serum was tested at a 1:5 dilution with tanned unsensitized sheep red cells, and a positive control serum of known titre was included in every batch of titrations. The sensitized cells were tested also against diluent to check for spontaneous agglutination. Sera were treated before titration with 0·1 M 2-mercaptoethanol for one hour at 37°C to obtain a partial identification of the class, IgG or IgM, of the reactive antibodies. The limitations of this procedure are acknowledged; however by gel filtration on Sephadex G-200, we could show that ME-sensitive antibodies to flagellin were present entirely in the IgM peak, and ME-resistant antibodies in the IgG peak (Rowley, Wistar & Mackay, 1969).

Using randomly selected sera, titres of antibody measured by TCH were compared with those obtained by bacterial immobilization (Ada *et al.*, 1964), using as antigen *Salmonella derby* which shares flagellar antigens but not somatic antigens with *S. adelaide*. Sera were inactivated, and dilutions were made in 1% foetal calf serum in saline. After addition of the bacterial suspension, titrations were left at room temperature for 30 min; the degree of immobilization was estimated by dark ground microscopy. The endpoint was the dilution of serum at which 80% immobilization was observed.

Statistical analysis of results

Geometric mean titres of total and ME-resistant antibody for each group of subjects at the selected timepoints were compared by Student's *t*-test. In calculating geometric means, titres of <5 were given the value of 1. Statistical calculations were made with a digital computer (IBM 7044).

Human subjects tested

The antibody response to flagellin was examined in 108 subjects from whom all the necessary blood samples had been taken, or when only the 10-week sample was lacking. They included fifty-four healthy persons (physicians, medical students, laboratory workers and healthy volunteers from elderly citizens' clubs) and fifty-four patients in hospital with illnesses not affecting the immune system. The harmlessness of the 5-μg dose of flagellin was established first in animals, and was then used to inject members of our laboratory group. The nature of the project was explained to all subjects before they were asked to participate. The groupings were as follows:

(1) *Healthy males* (twenty-seven), aged from 21 to 81 years, the mean age being 55 years.

(2) *Healthy females* (twenty-seven), aged from 19 to 83 years, the mean age being 56 years.

(3) *Hospital males* (twenty-seven), who were patients in hospital or in the out-patient clinic; one was receiving corticosteroids or immunosuppressive antimetabolites or antibiotics, most were suffering from gastroenterological or cardiovascular disorders, and they were aged from 21 to 83 years, the mean age being 58 years.

(4) *Hospital females* (twenty-seven), selected similarly to the 'hospital males' and aged from 17 to 85 years, the mean age being 58 years.

Men and women were compared by combining Groups 1 and 3 and Groups 2 and 4, and healthy subjects and those undergoing treatment were compared by combining Groups 1 and 2 and Groups 3 and 4. The effect of ageing was analysed by grouping the subjects into those aged less than 60 years (thirty-four), those aged 60–69 (thirty-eight) and those aged 70 years or over (thirty-six); the numbers of men and women in each group were similar.

The effect of the titres of 'natural' antibody before immunization was examined by grouping the subjects into those with a titre of 'natural' antibody greater than 40 (thirty-eight subjects, mean age 43 years), and those with a titre of 'natural' antibody of 40 or less (seventy subjects, mean age 65 years); this in effect also divided subjects according to age.

Sera of subjects other than those included in the above four groups were included in the segment of the work dealing with comparison of titration by TCH and bacterial immobilization.

The response to a second injection of flagellin was studied in twelve normal subjects, nine men and three women aged from 21 to 75 years, who were re-injected 1–19 months after the first injection.

RESULTS

Reaction to subcutaneous injection

The injection of 5 μg of flagellin resulted in a local area of induration and erythema, about 7 cm in diameter, which appeared after 3 hr and faded within 2 days. Occasionally a 1–2°C rise in temperature accompanied this reaction, or the axillary lymph nodes became palpable. The early appearance of this reaction suggested that it was a 'toxic' effect and not due to delayed hypersensitivity.

Antibody titre before immunization ('natural' antibody)

Antibody sensitive to mercaptoethanol was detected in the serum of ninety-three subjects (86%) before the injection of flagellin. Titres ranged from <5 to 2560 (mean 26). The age at which this 'natural' antibody appears is part of a subsequent paper, but it is very early—in some infants it was detected within 2 weeks of birth, and the titre was shown to fall gradually over the decades from 20 to 90 years (Rowley, Buchanan & Mackay, 1968).

Immune response of all subjects

The character of the immune response in 108 'immunologically normal' human subjects after injection of flagellin is shown in Fig. 1. Peak titres of antibody occurred 2 weeks after injection. Most sera contained predominantly ME-sensitive IgM antibody up to 10 weeks, but ME-resistant IgG antibody was produced very early and also reached a peak at 2 weeks. A complete change from IgM to IgG antibody production did not occur. However, eight subjects showed a predominantly IgG response in that there was less than a two dilution difference in the titre of total antibody and ME-resistant antibody. The mean peak titre for the 108 subjects was 2340 and the range was quite wide, from 1,280,000 to <5. The mean of the titres fell slowly over 10 weeks to 580.

*Immune response of males and females**

The immune response in men and women differed quantitatively when mean peak titres at 2 weeks were compared (Fig. 2). The peak for total antibody was significantly lower ($P<0{\cdot}05$) in men (mean 1490) than in women (mean 3670), but the peak titres of ME-resistant IgG antibody did not differ significantly.

* The data in this and the next section are based on combined groups (see 'Methods and subjects'). Analysis of the four groups separately according to sex and state of health showed trends similar to those recorded for the combined groups described above, but the groups were smaller and the differences did not reach statistical significance.

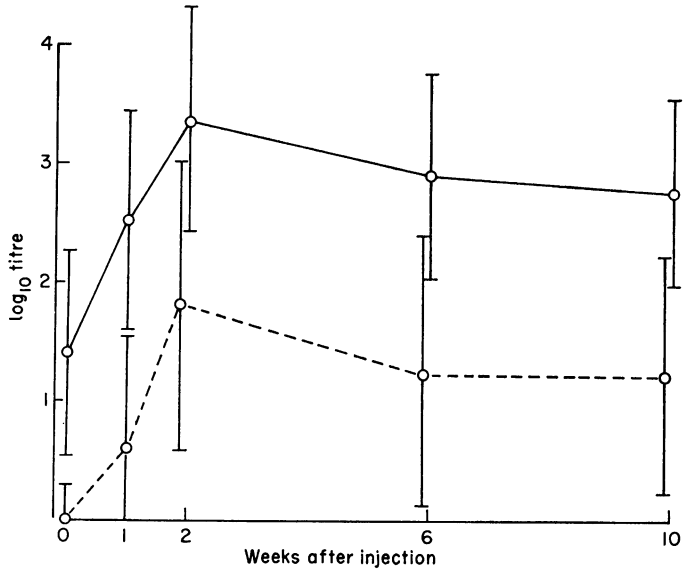

FIG. 1. Geometric mean titres of total antibody (——) and ME-resistant antibody (- - -) to flagellin in 108 healthy subjects and hospital patients. Vertical bars indicate 1 SD from the mean. The antibody present before injection and throughout the response was predominantly ME-sensitive, presumably IgM.

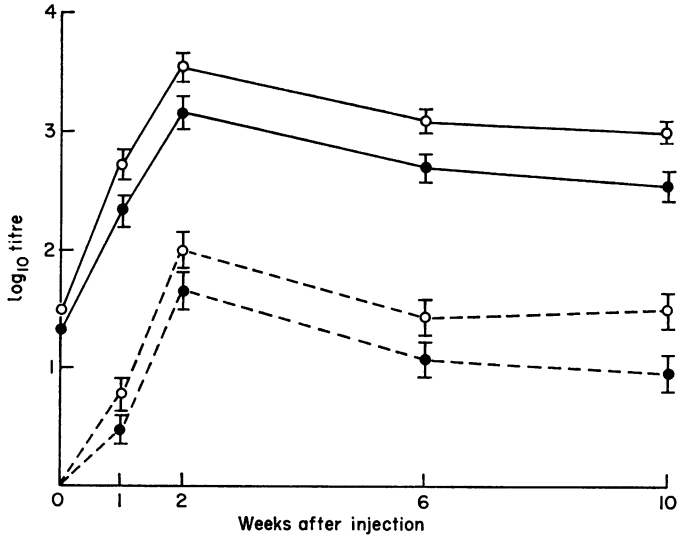

FIG. 2. Geometric mean titres of total antibody to flagellin were significantly higher ($P < 0.05$) in women (○) than men (●) at 1, 2, 6 and 10 weeks after injection of flagellin. Vertical bars in this and subsequent figures represent 1 SE of the mean. ——, Total antibody; - - -, ME resistant antibody.

Immune response of healthy subjects and hospital patients

The immune response of healthy subjects and patients attending or in hospital was compared by combining the groups of normal males and normal females, and 'hospital males' and 'hospital females', giving two groups of fifty-four men and fifty-four women. The peak for total antibody was significantly lower ($P<0{\cdot}02$) for 'hospital patients' (mean 1410) than for healthy subjects (mean 3890), but the peak titres of ME-resistant IgG antibody did not differ significantly (Fig. 3).

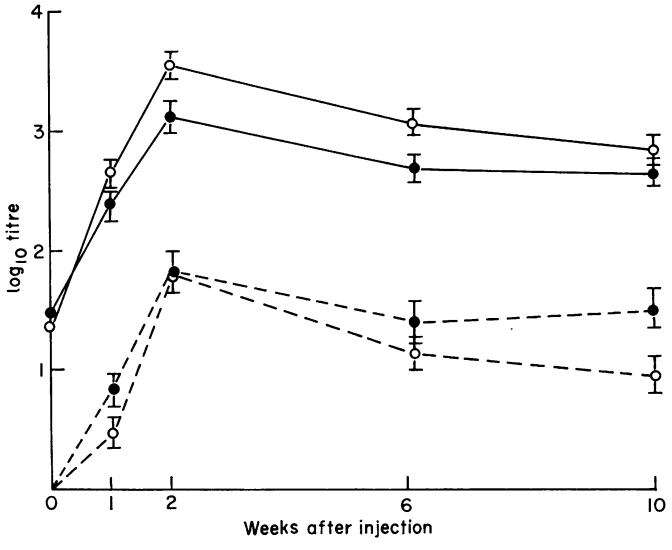

FIG. 3. Geometric mean titres of total antibody to flagellin were significantly higher ($P<0{\cdot}05$) in healthy subjects (○) than in those undergoing treatment in hospital (●) at 2 and 6 weeks after injection. ——, Total antibody; - - -, ME-resistant antibody.

Effect of age

Antibody-producing capacity according to age proved difficult to assess because of the relatively limited number of subjects tested. Titres of 'natural' antibody were significantly higher ($P<0{\cdot}001$) in the group aged less than 60 (mean 112) than in the two groups aged 60–69 years (mean 15) and 70 years and over (mean 12), corroborating the results from a previous study in which subjects were grouped by decades of age (Rowley *et al.*, 1968). However, after immunization mean titre of total antibody and ME-resistant antibody for each of the three age groups did not differ significantly (Fig. 4); moreover an analysis according to decades of age failed to show any influence of ageing on titres of antibody after immunization.

Effect of titre of 'natural' antibody

Grouping of subjects by their titre of 'natural' antibody tended to group them according to age, because the mean ages of the groups with pre-immunization titre $\leqslant 40$ and >40 were 65 and 43 years, respectively. Nevertheless the pre-immunization titre of 'natural' antibody did not influence the type or peak titre of antibody formed after immunization (Fig. 5), although titres fell more slowly in the group with high titres of 'natural' antibody.

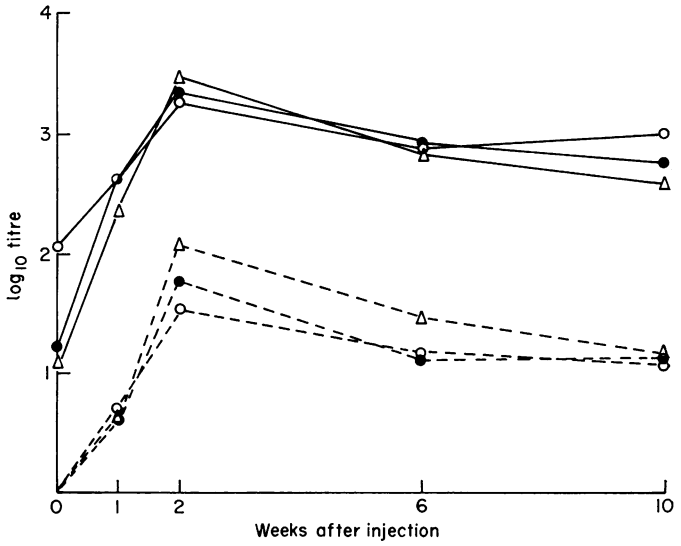

FIG. 4. Subjects aged 60–69 years and 70 years or over had significantly lower ($P < 0{\cdot}001$) titres of 'natural' antibody to flagellin than those aged < 60 years but for each of the three groups, titres of immune antibody did not differ significantly. ○, < 60 years; ●, 60–69 years; △, ⩾ 70 years; ——, Total antibody; - - -, ME-resistant antibody.

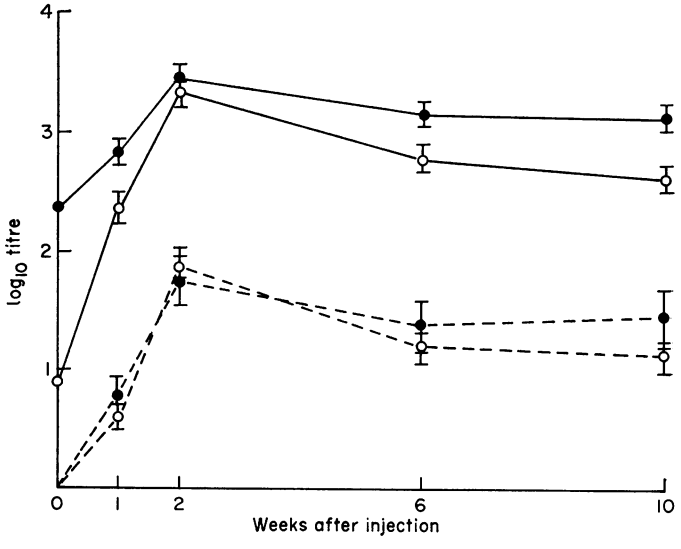

FIG. 5. The peak titres of immune antibody to flagellin did not differ in subjects grouped according to whether their titre of 'natural' antibody was 40 or less (○), or greater than 40 (●), but titres of total antibody fell more rapidly in those with low titres of 'natural' antibody. ——, Total antibody; - - -, ME-resistant antibody.

Titration of antibody by immobilization

Titres obtained by TCH were compared with those obtained by bacterial immobilization in 113 pre-immunization blood samples and in 233 randomly selected sera from 108 subjects tested 5–126 days after immunization. TCH proved to be the more sensitive procedure: it gave a higher incidence and higher titres of 'natural' antibody, and higher titres of immune antibody. However, there was no consistent relationship between results obtained by the two methods because in certain subjects titres by the two methods were similar (Fig. 6). This difference in sensitivity was apparently not related to the class of antibody present because neither immobilization nor TCH selectively detected IgM or IgG antibody.

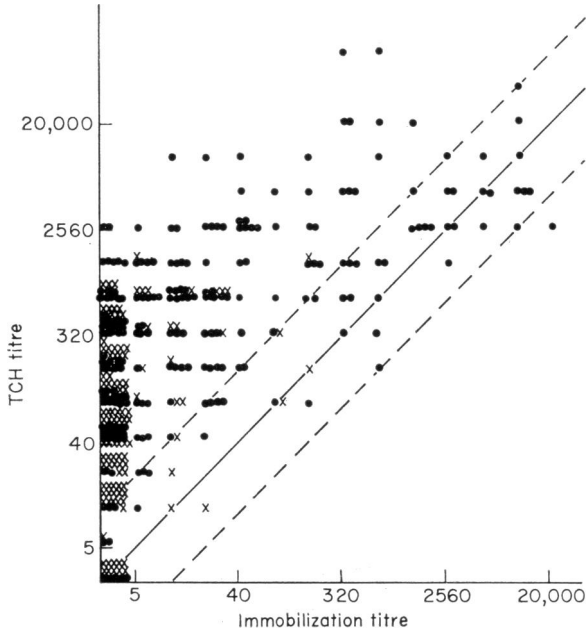

FIG. 6. Titres of antibody to flagellin measured by TCH and bacterial immobilization using *S. derby*. Titres were considered equivalent when the difference in titre was not greater than two dilutions (broken lines); in general TCH was the more sensitive. ×, Natural antibody; ●, immune antibody.

Secondary immune response

Although the timing of the response and the titres were similar after the first and second injections (Fig. 7), the characteristic feature after the second injection was the rapid change to production of IgG antibody. Thus 1 week after injection, only one subject showed a difference between total antibody and ME-resistant IgG antibody greater than one dilution, and at 2 weeks all subjects were apparently producing only ME-resistant antibody, presumably IgG.

DISCUSSION

Antibody producing capacity in man has seldom been studied systematically. Much of the information about factors which may influence the immune response in man has come either

from observations on the effects of various vaccines, where the major aim has been the rapid development of high titres of immune antibody, or from studies on antibody production by small numbers of patients with particular diseases and correspondingly small numbers of controls. In prophylactic immunization, antigens have been given with adjuvants (e.g. alum precipitated toxoids) and usually as a series of injections at short intervals, so preventing characterization of a simple primary response. Antibody-producing capacity in disease has been assessed using antigens such as pneumococcal polysaccharides (Larson & Tomlinson,

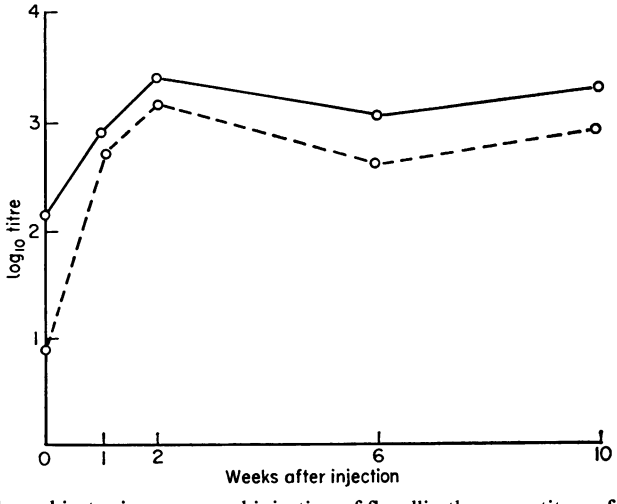

FIG. 7. In twelve subjects given a second injection of flagellin the mean titres of total antibody to flagellin were similar to those of the primary response, but the antibody was almost entirely ME-resistant and presumably IgG (cf. Fig. 1). ——, Total antibody; - - -, ME-resistant antibody.

1951), the Vi antigen of *E. coli* (Vanselow *et al.*, 1966), brucella organisms (Libánsky, 1965), plague vaccine (Adner *et al.*, 1966), blood group substances (Zingale *et al.*, 1963), bacteriophage ΦX174 (Cone & Uhr, 1964) and haemocyanin (Swanson & Schwartz, 1967). However, it is difficult to draw general conclusions from these studies for the following reasons. The groups tested were often small; the previous history of exposure to the antigen was uncertain; the antigens were complex; the methods of detecting antibody were in some cases cumbersome or insensitive; and there was no simple method for studying the class of antibody produced.

We chose flagellin as our test antigen for reasons previously stated (see 'Introduction'), including the considerable knowledge acquired in regard to the response in animals (Nossal *et al.*, 1964). There were in fact differences in the response of man and of animals to flagellin.

(1) The cutaneous reaction at the site of injection in man has not been observed in numerous studies in this Institute using mice, rats, guinea-pigs and rabbits.

(2) 'Natural' antibody to flagellin has been found only in man and the rabbit (Wistar, 1968).

(3) TCH and bacterial immobilization do not usually differ in sensitivity in animals as they do in man, except for the increased sensitivity of TCH in detecting the 'natural' antibody produced by rabbits and the antibody produced by rats in the first 5 days after primary immunization with flagella (Wistar, 1968).

(4) The primary response to flagellin in man was an IgM response and peak titres of antibody occurred 2 weeks after injection; however, in the rat the response was IgG and peak titres were not reached until 8 weeks after injection (Nossal *et al.*, 1964).

We could find few reports on the effects of sex differences on antibody production in animals or man. However, male cats produced less antibody than females after injection of *Brucella abortus* (Wilde & Naumann, 1960), and male mice produced less antibody than females after injection of sheep red cells (Stern & Davidson, 1955), non-viable tumour cells (Batchelor & Chapman, 1965) or bovine serum albumin (Terres, Morrison & Habicht, 1968). These observations are in agreement with our finding that after immunization of human subjects with flagellin, men produced significantly lower titres of antibody than women. This difference could be explained by the fact that IgM antibody is produced predominantly during the primary response to flagellin, and women have higher serum levels of IgM than do men (Butterworth, McClellan & Allansmith, 1967); however, the men in our study also produced less IgG antibody.

Ageing could not be shown to influence greatly the immune response to flagellin. Although titres of 'natural' antibody were lower in the groups aged more than 60 years, these subjects showed no delay in antibody production or lowering of peak titres of antibody. However, subjects with low titres of 'natural' antibody had a more rapid fall in titre, so that with ageing the capacity to maintain antibody production without stimulation may decline. It has been shown with ageing that there is a decrease in titre of 'natural' antibodies to sheep cell agglutinins (Paul & Bunnell, 1932), rabbit cell agglutinins (Friedberger, Bock & Furstenheim, 1929) and blood group agglutinins (Thomsen & Kettel, 1929). However, reports on immune responses in old age are scanty and contradictory in that Brenner, Waife & Wohl (1951) found a normal response to typhoid vaccine, whereas Virág & Kochar (1966) found lowered responses to tetanus anatoxin and LoSpalluto (1963) mentioned in discussion, without citing data, that there was a lowered response to paratyphoid bacteria.

Ill health and general debility could affect antibody producing capacity directly or, alternatively, an impaired capacity to produce antibodies could predispose to disease. Our 'hospital patients' had a significantly decreased capacity for antibody production as compared with healthy subjects, even though many of these 'hospital patients' were merely returning for follow-up visits to the out-patient clinic and were not very ill. Hence in any study which purports to show that impaired antibody-producing capacity is directly attributable to a particular disease, controls should include sick patients in hospital as well as healthy subjects.

We have described the response to one injection of flagellin as a primary response, despite the usual presence of 'natural' antibody in the serum, because it is only after a second injection of flagellin that there is evidence of 'immunological memory'. Thus the primary response was predominantly IgM, but after the second injection there was a rapid change to production of IgG. It is difficult to explain the presence of 'natural' antibody in humans to *S. adelaide* particularly as it may appear within the first few weeks of life. If, as is likely, it arises from contact early in life with a cross-reactive antigen, that antigen must be ubiquitous and frequently encountered.

We cannot yet state how applicable the response to flagellin would be in the investigation of individual patients. Firstly the range of responses of healthy subjects is wide, so that it is easier to compare groups of subjects rather than individuals. Secondly the procedure as done at present requires several weeks for a result, although the peak titre at 2 weeks appears to

give almost as much information as the character of the response over 10 weeks. Thirdly the response to primary immunization can be assessed only once, but this would apply to all test antigens.

We anticipate that the present techniques will have considerable potential for the practical study of the functional capacity of the immune system in man. They have proved suitable for examining factors which may influence antibody producing capacity in normal subjects, and could provide a standard procedure for the investigation of deficiency diseases, and for testing the effects of specific diseases or of therapeutic agents on antibody producing capacity. Moreover, the secondary response to flagellin might be used if there was need for serial testing of antibody-producing capacity in, for example, recipients of homografts or other patients receiving long term treatment with immunosuppressive drugs.

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