*Immunology*, 1972, **23**, 829.

# Studies on Immunological Paralysis

## IX. THE IMMUNOGENICITY AND TOLEROGENICITY OF LEVAN (POLYFRUCTOSE) IN MICE

J. J. MIRANDA*

*Department of Experimental Immunobiology, Wellcome Research Laboratories, Beckenham, Kent*

(*Received 27th March* 1972)

**Summary.** The immunological response to native levan (a fructose homopolymer with molecular weight $2 \times 10^7$) has been studied in (DBA/1 × CBA-T6T6)$F_1$ mice by passive haemagglutination and plaque-forming cell (PFC) assays. It resembles type-specific pneumococcal polysaccharide (SSS) in eliciting a prolonged humoral antibody response over a wide dose range (0·0001–100 $\mu$g) and in inducing long-lasting 'high zone' tolerance with a single injection (1 mg or more). Other similarities include an exclusively IgM response, independence of synergy with thymus-derived lymphocytes and absence of immunological memory. On the other hand, parallelism between serum haemagglutinin and PFC levels following all doses of antigen implies that higher immunizing doses of levan, unlike SSS, do not engage in peripheral neutralization of antibody. It was concluded from studying the fate of $^{14}$C-labelled levan that this was attributable to more rapid elimination from the circulation and subsequent slow metabolism of this polysaccharide. Levan also differs from SSS in inducing tolerance directly, without a detectable prior immune phase.

## INTRODUCTION

Polysaccharides have shown major differences in immunological behaviour as compared with protein antigens. Characteristics associated with them include: a very prolonged immune response which may be masked by antigen persistence, lack of immunological memory, induction of long-lasting tolerance with single injection in adult animals and the presence of 'reversibly tolerant' cells (see Howard, 1972). Pneumococcal polysaccharides, especially type 3 (SIII), have been most extensively studied and almost all the features quoted are related to work with this group of heteropolysaccharides. It seemed highly desirable to know if these findings represented generalizations applicable to polysaccharides as a whole.

The homopolysaccharide levan is one of the simplest natural antigens, being a high molecular weight polymer of fructose linked chiefly $\alpha$-(2→6). This substance in pure form

* Present address: Departmento de Microbiologia, Universidad Nacional de Trujillo, Facultad de Ciencias Medicas, Apartado No. 40, Trujillo, Peru, South America.

is able to induce an immune response in man (Allen and Kabat, 1957), but not in rabbits (Hehre, Genghof and Neill, 1945) unless it is associated with the whole bacteria which synthesize it. It has been suggested that one of the requirements for immunogenicity is the presence in a molecule of two different antigenic determinants (Bretscher and Cohn, 1970); if this were so, levan should not be immunogenic, as it is composed of identical units. We describe in this and the following communication, some of the characteristics of immunity and tolerance to levan in mice. Important dissimilarities from the corresponding responses to SIII have emerged.

## MATERIALS AND METHODS

*Animals*

(DBA/1 × CBA-T6T6)$F_1$ mice of either sex, with body weight between 20–25 g, were used throughout the experiments.

*Antigens*

Levan was isolated from two bacterial sources: *Corynebacterium levaniformis* (NCBI 9659) and *Enterobacter levanicum* (NCBI 9966), according to the technique of Hehre *et al.* (1945). Estimation of levan in these preparations was made by the Dische Borenfreund method (see Kabat and Mayer, 1961). The *C. levaniformis* preparation had an average molecular weight (mol. wt) of $2 \times 10^7$ and was 97 per cent pure, whilst the *E. levanicum* preparation had a mol. wt of $1{\cdot}6 \times 10^7$ and was 98 per cent pure. (I am indebted to Dr H. Zola for the mol. wt determinations.)

*C. levaniformis* levan labelled with $^{14}C$ was prepared using the same procedure, but adding 0·5 mCi of uniformly labelled $^{14}C$ (Radiochemical Centre, Amersham) to a volume of 500 ml medium. The culture was incubated for 4 days at room temperature. $CO_2$ from this culture was trapped as described by Howard, Christie, Jacob and Elson (1970). The activity of this preparation was 75,000 counts/mg/min.

*Immunization*

*C. levaniformis* levan was dissolved in 0·15 M NaCl and injected i.v. into the tail vein in a volume of 0·5 ml.

*Antibody determinations*

The immune response to levan was studied by two means: serum titration by passive haemagglutination and enumeration of antibody-secreting cells by a direct Jerne plaque assay. Levan from *E. levanicum* only was used for sensitizing red cells for both techniques, to exclude the unlikely possibility of interference from minimal impurity in the *C. levaniformis* product. (The two levans gave similar complement fixation titres with a rabbit anti-levan serum.)

A. Passive haemagglutination was carried out using syngeneic mouse red blood cells sensitized with levan as described by Hirata and Brandiss (1968), except that glutaraldehyde was used for stabilizing the cells (Bing, Weyand and Stavitsky, 1967) instead of pyruvic aldehyde. It was found that the optimal amount of levan for sensitization was 1 mg per 0·1 ml packed cells. Positive control 'serum' consisted of purified J 606 myeloma protein with antilevan specificity, kindly provided by Dr Melvin Cohn. At 1 mg/ml concentration it gave a $\log_2$ haemagglutinin titre of 8. Rabbit anti-levan serum was also

used in some experiments and had been prepared as follows: a rabbit was immunized i.v. twice weekly with *E. levanicum* vaccine ($2 \times 10^8$ bacteria per ml)—0·5, 1·0 and 2·0 ml per injection during weeks 1, 2 and 3. The final $\log_2$ haemagglutinin titre attained was 10.

B. Plaque-forming cells (PFCs) were studied by the technique of Jerne, Nordin and Henry (1963) with the modifications described by Howard, Christie and Courtenay (1971a). Sheep erythrocytes were sensitized with O-Stearoyl levan prepared according to Hämmerling and Westphal (1967). This preparation contained 4 per cent Stearoyl and 10 $\mu$g was the optimal amount for sensitizing 0·1 ml of packed cells.

It should be recorded briefly that the following attempted sensitization procedures were found wholly or partly ineffective for the PFC assay: (a) incubation of cells with levan alone, NaOH treated levan, supernatant of *E. levanicum* culture (with or without NaOH treatment); (b) treatment with Concanavalin A or $CrCl_3$.

For investigation of indirect PFCs, 2 ml of rabbit anti-mouse Fab serum (provided by Dr P. del Guercio) diluted 1/2000 was flooded on the plate, after 1-hour incubation. The plates were incubated for another hour before C was added. The 1/2000 dilution of rabbit anti-mouse Fab serum was selected as it developed indirect plaques to SRBC with minimal inhibition.

*Sephadex G-200 filtration*

Two millilitres of pooled serum was applied to an 80 × 1·75-cm Sephadex G-200 column equilibrated with Tris-HCl 0·1 M buffer (pH 8·2) and NaCl 0·2 M. Fractions were collected in volumes of 2 ml and the optical densities read at 280 m$\mu$ in a Beckman DB Spectrophotometer.

*Irradiation*

Mice were irradiated using a $^{60}$Co source with a dose rate of approximately 30 R/minute and a focal distance of 18 cm.

*Cell suspensions*

Bone-marrow (BM) cells were obtained by flushing out the tibia and femur with medium 199. The cells were dispersed by passing repeatedly through the needle. Cell suspensions were obtained from the thymus by dissociation with a loose-fitting ground glass homogenizer. For repopulation experiments, $4 \times 10^7$ viable BM cells and $10^8$ viable thymus cells were injected i.v. into 900 R-irradiated recipients.

Treatment of BM cell suspensions with anti-$\theta$ serum and complement for elimination of T cells was carried out using the same method and batch of serum as described by Howard, Christie, Courtenay, Leuchars and Davies (1971b).

*Clearance of [$^{14}$C]levan from the circulation*

Mice were injected with [$^{14}$C]levan i.v. and subsequently bled from the retro-orbital plexus at different intervals. The general procedure and calculations followed those described by Howard *et al.* (1970).

## RESULTS

### SPECIFICITY OF ASSAYS

The possibility was first excluded that the preparations of levan from *C. levaniformis* and

*E. levanicum* might contain a contaminating substance common to the two species which would interfere in the assays. A group of mice was injected with 100 μg of *E. levanicum* levan and 10 days later the animals were bled and the serum pooled. Aliquots were absorbed with 1/3 volume of washed packed bacteria—*C. levaniformis* or *E. levanicum* containing levan or the same bacteria devoid of levan. (The latter were obtained by growth in sucrose-free medium.) The results of haemagglutination titres before and after absorption are given in Table 1, showing that only bacteria containing levan (either *C. levaniformis* or *E. levanicum*) were able to inhibit the haemagglutinating antibody.

Furthermore, when mice were injected with levan-positive *E. levanicum* vaccine ($10^9$ bacteria in 0·5 ml) and the response assayed by enumeration of the antibody-forming cells, 10 days later, haemolytic plaques were inhibited by low concentrations (5 μg/ml) of *C. levaniformis* levan in the gel. These results indicate that in both systems only the response to levan is measured.

TABLE 1

EFFECT OF ABSORPTION WITH *E. levanicum* AND *C. levaniformis* ON THE HAEMAGGLUTININ TITRE ($LOG_2$) OF SERUM FROM MICE IMMUNIZED WITH 100 μg *E. levanicum* LEVAN

| Titre before absorption | Titre after absorption with | | | |
|---|---|---|---|---|
| | *E. levanicum* | | *C. levaniformis* | |
| | Levan* | Levan† | Levan* | Levan† |
| 7 | 1 | 6 | 1 | 7 |

* Bacteria containing levan. † Levan-free bacteria.

## DOSE RESPONSE

Groups of mice were injected i.v. with amounts of levan ranging from $10^{-4}$ to $10^4$ μg and 4 days later estimations of serum haemagglutinin titres and direct PFC counts in the spleens were made on individual animals. The results presented in Fig. 1 show that levan is immunogenic in mice. As little as $10^{-4}$ μg induced a detectable response, whilst 10 μg was the optimal immunizing dose assessed at both humoral and cellular levels. When the dose was increased above $10^2$ μg tolerance was induced. The strict parallelism between the humoral antibody and PFC responses over the whole dose range is noteworthy as it implies that levan, unlike SIII, does not effect peripheral neutralization of antibody when given in higher immunizing doses.

To ensure that the observed unresponsiveness after $10^3$–$10^4$ μg levan was due to central failure and not to the presence of 'masking' antigen, spleen cell suspensions from tolerant animals were mixed with those from immune mice and PFC assays performed. The number of plaques detectable was not inhibited by the admixture of 'tolerant' with immune cells.

The lack of response following high doses of levan represents specific tolerance and is not due to any toxic effect of the polysaccharide. Groups of five mice were immunized with $4 \times 10^8$ sheep erythrocytes i.v., with or without injection of 10 mg levan 10 days previously. There was no difference in the PFC response of the two groups 4 days later—geometric

means of 71,290 and 90,990 PFCs per spleen in the normal and levan-treated groups respectively ($\log_{10} \pm$standard error: $4{\cdot}853 \pm 0{\cdot}033$ and $4{\cdot}959 \pm 0{\cdot}088$).

### THE IMMUNE RESPONSE AT VARYING INTERVALS

Haemagglutinin titres and spleen PFC counts were investigated at different intervals after a single injection of 1 and 100 $\mu$g and 1 and 10 mg. The results expressed in Figs 2 and 3 show that the response to 1 $\mu$g is more rapid in onset than that to 100 $\mu$g. The peak PFC response is detectable at day 4 after levan injection, and thereafter declines slowly, remaining elevated at 6 weeks.

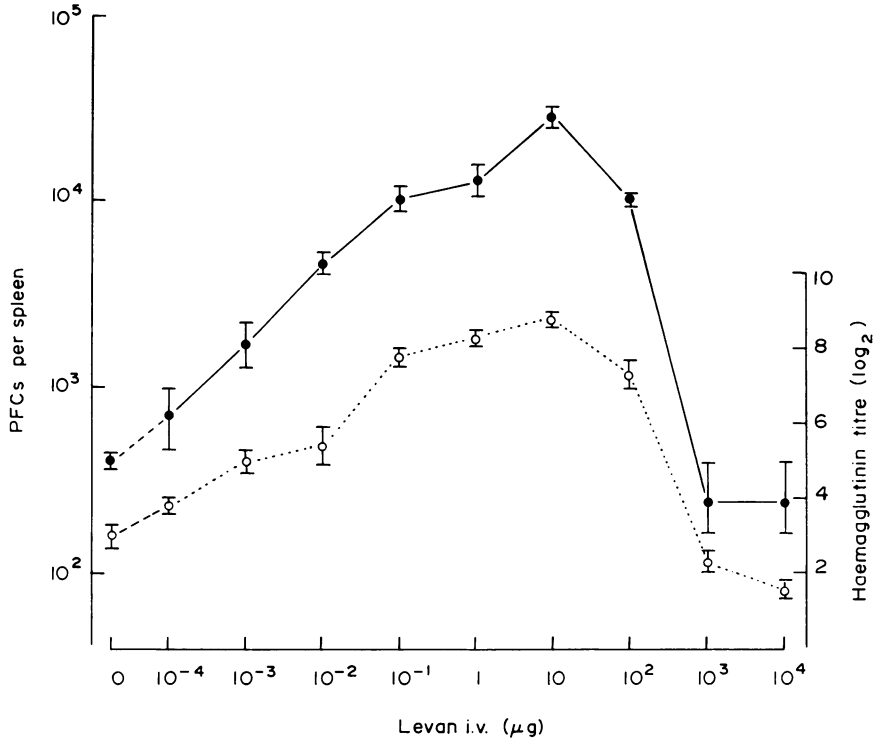


FIG. 1. Mean anti-levan haemagglutinin titres and mean direct PFC levels in the spleen 4 days after i.v. injection of various doses of levan. (Standard errors indicated.) No. of readings per point varied between 3 and 25. (●) PFCs; (○) haemagglutinin.

Characteristics of the immune tolerance induced by 1–10 mg levan will be discussed elsewhere (Miranda, Zola and Howard, 1972). It should be noted here, however, that no early immune response was detectable preceding the PFC suppression induced by 1 and 10 mg levan. The spontaneous rise of haemagglutinin titre at day 40 in mice injected with 1 mg of levan and the lack of PFC response in their spleens could be explained by antibody being synthesized in other sites during the early stage of recovery from tolerance.

### CLASS OF IMMUNOGLOBULIN WITH ANTILEVAN SPECIFICITY

The nature of the antibody synthesized in the response to levan was studied by gel filtration and by using rabbit anti-mouse Fab serum for attempted development of indirect plaques in the PFC assay.

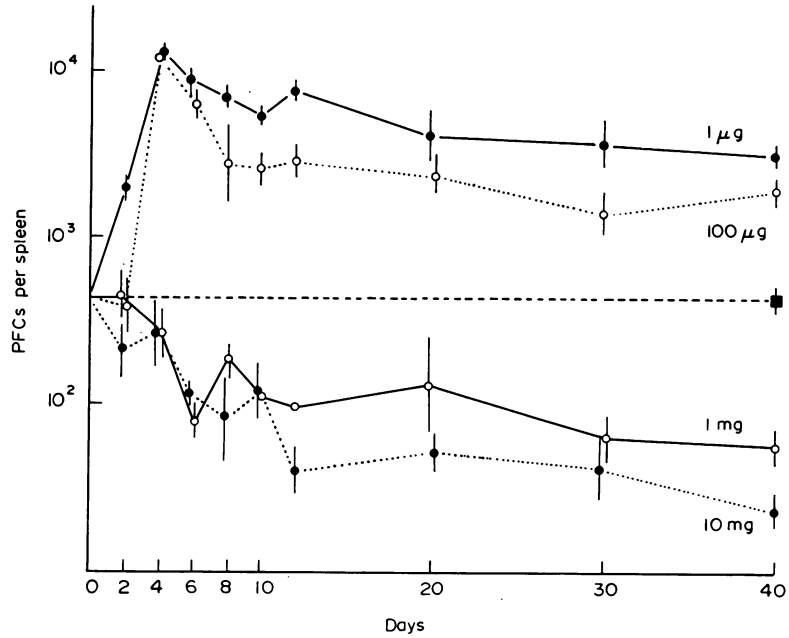


FIG. 2. A comparison of the direct PFC levels in the spleen at various times after different doses of levan. (Mean background ± SE on twenty-five mice indicated.) Four mice per point.

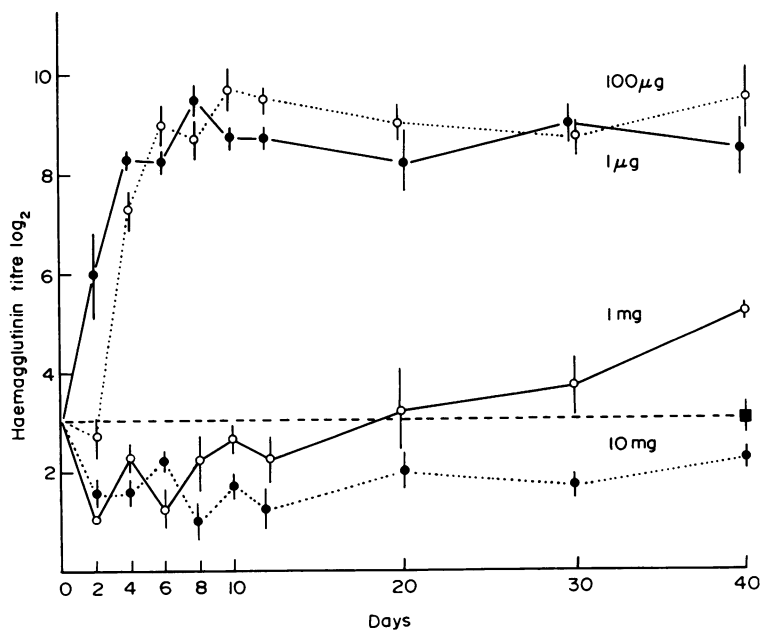


FIG. 3. A comparison of the haemagglutinin levels at various times after different doses of levan. (Mean background ± SE on twenty-five mice indicated.) Four mice per point.

Batches of mice were immunized with 1 or 100 $\mu$g of levan and their group serum pooled 6 days later for fractionation by Sephadex G-200. Haemagglutination titrations were carried out with the different fractions, which were collected in 2-ml volumes. Positive activity was detectable only in fractions from the first (IgM) peak and was wholly inactivated by treatment with 2-mercaptoethanol (Fig. 4). The same result was obtained when either 1 or 100 $\mu$g of levan was used for immunization.

Indirect PFCs were sought in the spleen at various intervals after different immunizing doses of levan (Table 2). No indirect PFCs could be detected, and furthermore, when greater concentrations of developing serum were employed, complete inhibition of the haemolytic plaques resulted. These results imply that only IgM is produced in the immune response to levan.

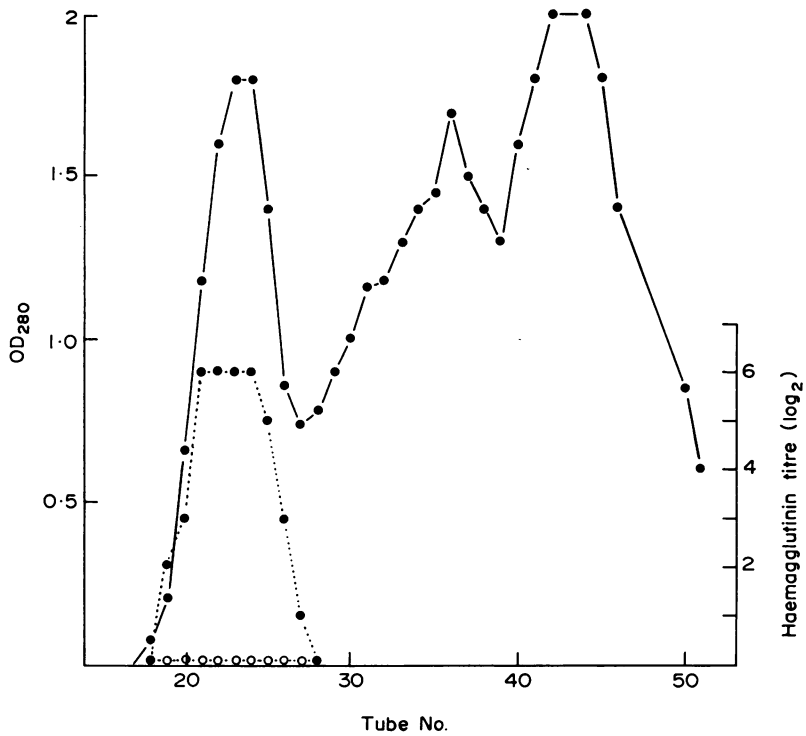


FIG. 4. Fractionation by Sephadex G-200 gel filtration of pooled serum from mice immunized with 1 $\mu$g levan 6 days previously (●—●). The localization of anti-levan haemagglutinating activity in the IgM peak is shown (● · · · · ●) together with its mercaptoethanol sensitivity (○ · · · · ○).

## SPECIFIC INHIBITION OF PFCS

It was found by Andersson (1970), that the avidity of the antibody secreted by individual antibody-forming cells is correlated inversely with the amount of antigen required to inhibit the haemolytic plaques they produce. The avidity of the antibody produced in response to levan was investigated by specific inhibition of direct PFCs when varying amounts of levan were incorporated in the gel, following the description by Andersson (1970). The neutralization curve in relation to dose of levan added (Fig. 5) is far steeper than those for multideterminant serum protein antigens found by Andersson,

TABLE 2

FAILURE TO DEMONSTRATE INDIRECT PFCs IN THE SPLEEN AT DIFFERENT INTERVALS AFTER VARIOUS DOSES OF LEVAN

| Interval between injection of levan and assay (days) | Dose of levan i.v. ($\mu$g) | PFCs per spleen* | |
|---|---|---|---|
| | | Without developing serum | With developing serum† |
| 4 | 1 | 4·018±0·063 (10420) | 3·948±0·026 (8872) |
| | 100 | 4·056±0·006 (11380) | 3·978±0·01 (9506) |
| | 1000 | 2·005±0·05 (101) | 1·780±0·12 (60) |
| 10 | 1 | 3·731±0·05 (5383) | 3·711±0·05 (5140) |
| | 100 | 3·418±0·102 (2618) | 3·277±0·119 (1892) |
| | 1000 | 2·049±0·049 (111) | 1·948±0·05 (88) |
| 30 | 1 | 3·576±0·145 (3681) | 3·561±0·146 (3639) |
| | 100 | 3·154±0·138 (1426) | 2·981±0·171 (957) |
| | 1000 | 1·802±0·144 (63) | 0 |

* Geometric means of four mice ($\log_{10}$ ± SE) with antilog in parentheses.
† Rabbit anti-mouse Fab, 1/2,000 dilution.

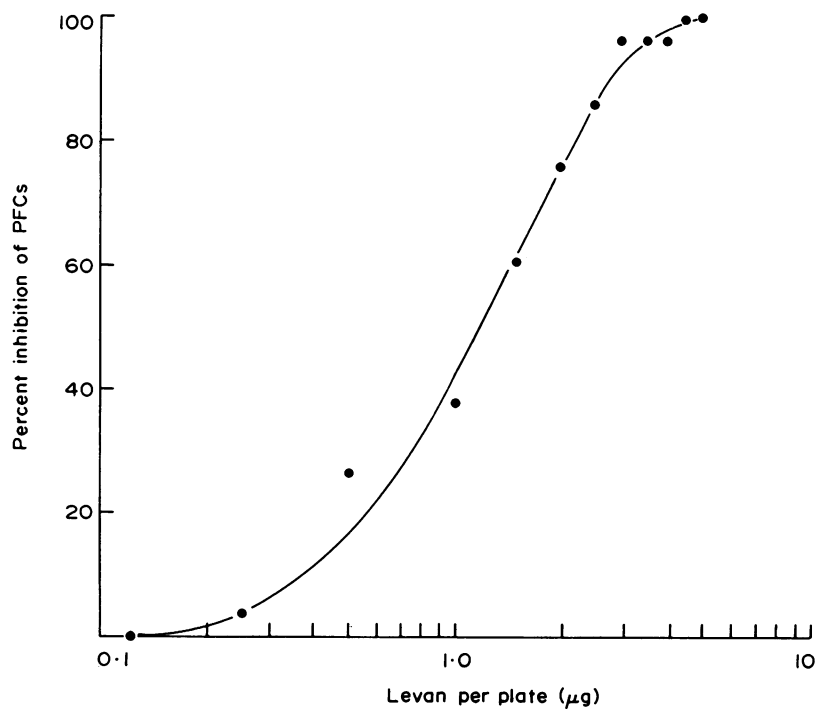


FIG. 5. Specific inhibition of anti-levan PFCs by the incorporation of levan in the gel. Spleen cells were from mice injected with 10 $\mu$g levan 4 days previously.

indicating that mouse antilevan antibody has relatively uniform avidity in accordance with the unideterminant nature of the antigen. The 50 per cent neutralization dose was 1·2 μg levan per plate.

### SECONDARY RESPONSE

To ascertain whether levan is capable of inducing IgM memory, groups of mice were injected with a small dose (0·001 μg) for priming and a higher one (10 μg) as second injection. The intervals between these injections were 2, 4 and 30 days. Other groups of mice were injected with single doses of 0·001 or 10 μg alone at times corresponding to priming or challenge in the experimental groups. PFC assay was carried out 3 days after

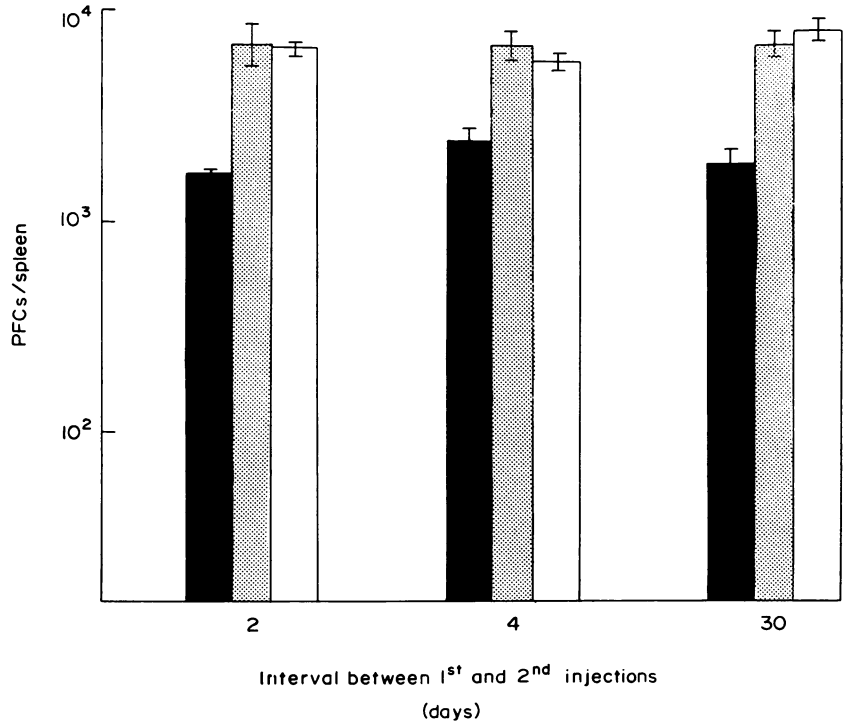


FIG. 6. Failure to develop immunological memory with levan. Direct PFC responses (± SE) in groups of ten mice injected with 0·001 μg levan and challenged with 10 μg at various times subsequently (open columns). Corresponding groups of three and seven mice receiving only 1st (solid columns) and 2nd (stripped columns) injections respectively are shown.

the second injection so as to detect any possible early rise in the response. The results presented in Fig. 6 show that within the limits of dosage and time studied, levan resembles other polysaccharides in not inducing immunological memory.

### THYMUS-INDEPENDENCE OF THE IMMUNE RESPONSE TO LEVAN

As levan is one of the simplest known antigens, experiments were performed to see whether it resembled other polymeric antigens in evoking an immune response which is independent of synergy with T lymphocytes. Lethally-irradiated mice were repopulated with large doses of syngeneic bone marrow or thymus cells or both and then challenged

with 10 μg levan. The results of these experiments (Table 3) show that recipients of bone marrow cells alone are able to respond fully to the challenge and that the addition of thymus cells does not confer any co-operative augmentation of the response.

To exclude the possibility that the dose of levan used in these experiments was sufficiently high to obliterate any potential helper effect of thymus cells, a much smaller dose (0·1 μg) was also tested (Expt. 4 in Table 3). A feebler response was obtained in both groups without any significant difference between the number of PFCs. Since bone marrow cell

TABLE 3

PFC RESPONSE IN THE SPLEEN TO LEVAN IN LETHALLY-IRRADIATED MICE REPOPULATED WITH SYNGENEIC THYMUS CELLS, BONE MARROW CELLS OR BOTH

| Injected i.v. into 900 R-irradiated mice | | | PFCs per spleen† | | | |
|---|---|---|---|---|---|---|
| Thymus cells ($10^8$) | B.M. cells ($4\times10^7$) | Levan* | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 |
| + | + | — | 2·590 ± 0·105 (389) | 2·940 ± 0·11 (871) | 2·977 ± 0·085 (948) | 2·977 ± 0·085 (948) |
| + | — | + | 1·855 ± 0·198 (71) | N.T. | 2.103 ± 0·035 (126) | 2·271 ± 0·052 (186) |
| — | + | + | 3·597 ± 0·136 (3954) | 3·819 ± 0·043 (6592) | 3·500 ± 0·43 (3162) | 3·221 ± 0·045 (1663) |
| + | + | + | 3·649 ± 0·057 (4457) | 4·049 ± 0·054 (11190) | 3·526 ± 0·054 (3357) | 3·181 ± 0·042 (1517) |

* 10 μg of levan was injected in Expts 1–3 and 0·1 μg in Expt. 4.
† Geometric means of four mice ($\log_{10} \pm$ SE) with antilog in parentheses. (Three mice in Expt. 1.)
N.T. = not tested.

TABLE 4

FAILURE OF *in vitro* TREATMENT WITH ANTI-θ SERUM TO ABOLISH ABILITY OF BONE MARROW CELLS TO TRANSFER LEVAN-RESPONSIVENESS TO 900 R-IRRADIATED MICE

| Pre-treatment of bone marrow cells injected | 10 μg levan i.v. | PFCs per spleen* |
|---|---|---|
| None | — | 2·940 ± 0·11 (871) |
| None | + | 3·819 ± 0·043 (6592) |
| 1 : 2 Normal AKR serum + C | + | 3·883 ± 0·051 (7638) |
| 1 : 2 Anti-θ serum + C | + | 3·852 ± 0·044 (7112) |

* Geometric means of four mice ($\log_{10} \pm$ SE) with antilog in parentheses.

suspensions contain a small number of thymus-derived cells that might conceivably be numerically adequate to 'co-operate' with B lymphocytes in response to levan, suspensions treated with anti-θ serum and complement so as to destroy T cells were also tested. The results expressed in Table 4 show that this treatment did not affect the capability of bone marrow cells to confer responsiveness to levan.

We conclude from these experiments, that immunity to levan represents a direct B cell response in which T cells do not play any co-operative role in the induction phase.

CLEARANCE OF [$^{14}$C]LEVAN FROM THE CIRCULATION

As levan appeared to differ from SIII in not producing any 'treadmill' neutralization of antibody, its fate was studied in mice with regard to elimination from the circulation and possible metabolism. A tolerizing dose (1 mg) of $^{14}$C-labelled levan was injected i.v. into a group of ten mice which were bled after 15 minutes, 2, 4, 6, 10, 24, 48 and 72 hours. The $\beta$ emission counts of the serum samples were counted and the residual radioactivity expressed as a percentage of the initial level (Fig. 7). Approximately 50 per cent clearance took place within 2 hours and 98 per cent by 24 hours. Only 0·3 per cent remained in the circulation after 72 hours. A preliminary experiment to see whether levan is metabolized in the mouse was kindly performed by Dr R. Nimmo-Smith, Department of Drug Metabolism, Wellcome Research Laboratories. Two mice were injected i.p. with 20 mg $^{14}$C-labelled levan and the fate of the $^{14}$C studied with an all-

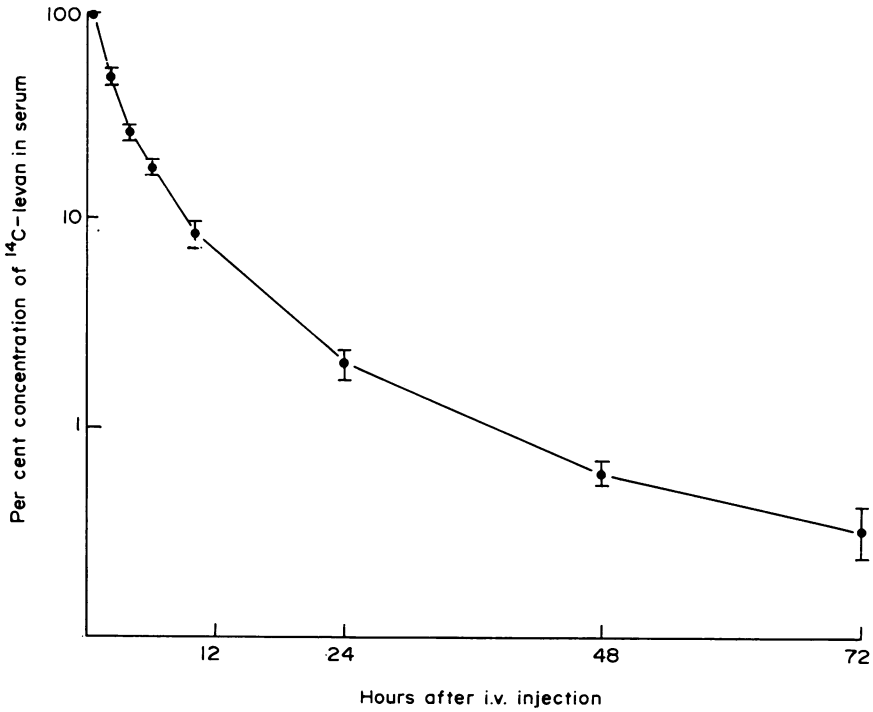


FIG. 7. Mean rate of clearance from the circulation of 1 mg [$^{14}$C]levan injected i.v. in ten normal mice. (Standard errors indicated.)

glass metabolism cage. It was found that within 3 days, 5·7 per cent of the isotope was expired as $CO_2$ in the breath of these mice, whilst a further 7·8 and 4·6 per cents were excreted in urine and faeces respectively. The conclusion from these experiments is that levan is metabolized slowly by mice.

## DISCUSSION

The injection of levan alone in mice will induce a humoral immune response or tolerance according to dosage. Serum antibody levels remain constant for a long time after a wide

range of immunizing doses, whilst a single injection of 1 mg or more will induce a prolonged state of tolerance. Similar characteristics of response have been described for other polysaccharides (see Kabat and Mayer, 1961; Howard, 1971) as has the synthesis of IgM alone, absence of immunological memory and T-cell independence, which will be commented on briefly.

The range separating an immunizing from a tolerogenic dose of levan is narrow (100 $\mu$g–1 mg), like other polysaccharides (Howard *et al.*, 1971a; Baker, Stashak, Amsbaugh and Prescott, 1971; Britton, 1969), but in contrast with serum protein antigens. This difference could be explained on the basis that antigens with homopolymeric structure induce the synthesis solely of IgM antibodies with uniform avidity. The receptor on the potential antibody-producing cells would therefore be multivalent and a relatively small increase in the dose of an antigen composed of uniform repeating antigenic determinants could be sufficient for binding to the number of receptor sites on these cells necessary for inducing tolerance.

A major regulating factor in the immune response to many antigens (e.g. heterologous erythrocytes and serum proteins) is the co-operative or helper effect of thymus-derived (T) cells (Claman, Chaperon and Triplett, 1966; Miller and Mitchell, 1968; Chiller, Habicht and Weigle, 1970; Unanue, 1970). Other immunogens are wholly independent of T cell synergy (Andersson and Blomgren, 1971; Humphrey, Parrot and East, 1964; Howard *et al.*, 1971b). The main common characteristic of substances of this category described to date is that structurally they are all polymeric, so that association between the antigen and corresponding B lymphocytes is increased (see Mitchison, 1971; Möller, 1970), thereby obviating the need for T cells to amplify the immune response. It seemed likely that levan, as a simple polymer of fructose, would be equally thymus-independent. This was found to be the case, since in the attempted co-operation experiments thymus cells did not enhance the response of bone marrow cells when used in numbers which will demonstrate synergy towards other antigens. Moreover, treatment of bone marrow cells with anti-$\theta$ serum and complement so as to destroy any T cells before transfer did not affect the response. The possibility that the dose of levan used in these experiments was sufficiently high to overcome any potential helper effect of T cells (Taylor and Wortis, 1968; Playfair and Purves, 1971) was excluded, since when a low dose of antigen was used, it only induced a similar feeble PFC response in mice repopulated with either a mixture of thymus and bone marrow cells or with the latter alone.

Polysaccharides have been generally described as antigens which are, in contrast with proteins deficient in the capacity for inducing immunological memory (e.g. Byfield, Christie, Kotlarski, Miranda and Salerno, 1972). IgM memory has been found with certain antigens (Nossal, Austin and Ada, 1965; Sercarz and Byers, 1967; Byfield and Sercarz, 1969), using a small dose for priming and a large dose for the second injection. The enhanced response is detectable only for a short period after priming—as early as 30 hours, but usually disappearing after 4 weeks. As levan stimulates a purely 19S response, we sought evidence of similar IgM memory, but without success. This might have been due to the presence of circulating antibodies that compete with cell-bound antibody receptors for antigen, thereby impairing the secondary response. It seems to be a general characteristic, however, that thymus-independent antigens do not induce immunological memory, which is carried mainly by the T cell population (Möller, 1970; Cunningham, 1969; Raff, 1970).

In spite of these characteristic polysaccharide attributes, two major differences between

the responses to levan and SIII have emerged from the present investigation. First, the effect of optimal immunizing doses of levan was not masked by peripheral neutralization with persisting antigen. Serum antibody titres and PFC levels in the spleen were directly correlated, whereas haemagglutinating antibody is neutralized by recirculating SIII when higher doses are used for immunization (Howard *et al.*, 1970, 1971a). The absence of a 'treadmill' effect with levan is most probably explained by much more rapid elimination of antigen from the circulation as compared with SIII, together with its subsequent metabolism. Clearance of the same high dose of both polysaccharides injected i.v. at 24, 48 and 72 hours was found to be 98, 99·4 and 99·7 per cent for levan, as compared with 52, 71 and 82 per cent for SIII (data from Howard *et al.*, 1970).

The second important dissimilarity between these polysaccharides concerns the induction of tolerance. Whereas this is consistently preceded by a detectable immune phase in the case of SIII (Siskind and Howard, 1966; Howard *et al.*, 1971a), no analogous PFC response was detectable with levan. Furthermore, although both models of tolerance are similarly long-lasting, that with levan is far more rapid in its onset and differs profoundly from the SIII system in being totally irreversible on cell transfer (cf. Howard, Christie and Courtenay, 1972). These experiments are described and their theoretical significance considered in the succeeding paper (Miranda *et al.*, 1972).

## ACKNOWLEDGMENTS

I wish to thank Dr J. G. Howard for his encouragement and helpful discussions, Mr G. H. Christie, Mrs B. M. Courtenay and Miss A. Middleton for their technical advice, Dr R. Nimmo-Smith for performing the levan metabolism experiment and the Chemistry Department, Wellcome Research Laboratories for preparing O-Stearoyl levan.

## REFERENCES

ALLEN, P. and KABAT, E. A. (1957). 'Studies on the capacity of some polysaccharides to elicit antibody formation in man.' *J. exp. Med.*, **105**, 383.

ANDERSSON, B. (1970). 'Studies on the regulation of avidity at the level of the single antibody-forming cell.' *J. exp. Med.*, **132**, 77.

ANDERSSON, B. and BROMGREN, H. (1971). 'Evidence for thymus independent humoral antibody production in mice against polyvinyl pyrrolidone and *E. coli* lipopolysaccharide.' *Cell. Immunol.*, **2**, 411.

BAKER, P. J., STASHAK, P. W., AMSBAUGH, F. and PRESCOTT, B. (1971). 'Characteristics of the antibody response to type III pneumococcal polysaccharide at the cellular level. I. Dose response studies and the effect of prior immunization on the magnitude of the antibody response.' *Immunology*, **20**, 469.

BING, D. H., WAYAND, J. G. M. and STAVITSKY, A. B. (1967). 'Haemagglutination with aldehyde fixed erythrocytes for assay of antigens and antibodies.' *Proc. Soc. exp. Biol. (N.Y.)*, **124**, 1166.

BRETSCHER, P. A. and COHN, M. (1970). 'A theory of self-nonself discrimination.' *Science*, **169**, 1042.

BRITTON, S. (1969). 'Regulation of antibody synthesis against *Escherichia coli* endotoxin. II. Specificity, dose requirements and duration of paralysis induced in adult mice.' *Immunology*, **16**, 513.

BYFIELD, P., CHRISTIE, G. H., KOTLARSKI, I., MIRANDA, J. J. and SALERNO, A. (1972). 'Are T cells necessary and/or sufficient for generation of B cell memory?' *Europ. J. Immunol.* (In press).

BYFIELD, P. and SERCARZ, E. (1969). 'The X-Y-Z scheme of immunocyte maturation. VII. Cell division and the establishment of short term IgM memory.' *J. exp. Med.*, **129**, 897.

CHILLER, J. M., HABICHT, G. S. and WEIGLE, W. O. (1970). 'Cellular sites of immunological unresponsiveness.' *Proc. nat. Acad. Sci.*, **65**, 551.

CLAMAN, H. N., CHAPERON, E. A. and TRIPLETT, R. F. (1966). 'Thymus-marrow cell combinations. Synergism in antibody production.' *Proc. Soc. exp. Biol. (N.Y.)*, **122**, 1167.

CUNNINGHAM, A. J. (1969). 'Studies on the cellular basis of IgM immunological memory.' *Immunology*, **16**, 621.

HÄMMERLING, V. and WESTPHAL, O. (1967). 'Synthesis and use of O-stearoyl polysaccharides in passive hemagglutination and hemolysis.' *Europ. J. Biochem.*, **1**, 46.

HEHRE, E. J., GENGHOF, D. S. and NEILL, J. M. (1945). 'Serological reaction of two bacterial levans.' *J. Immunol.*, **51**, 5.

HIRATA, A. A. and BRANDISS, M. W. (1968). 'Passive hemagglutination procedures for protein and polysaccharide antigens using erythrocytes stabilized by aldehydes.' *J. Immunol.*, **100**, 641.

HOWARD, J. G. (1972). 'Cellular events in the induction and loss of tolerance to pneumococcal polysac-

charides.' *Transplant. Rev.*, **8**, 50.

HOWARD, J. G., CHRISTIE, G. H. and COURTENAY, B. M. (1971a). 'Studies on immunological paralysis. IV. The relative contributions of continuous antibody neutralisation and central inhibition to paralysis with type III pneumococcal polysaccharide.' *Proc. roy. Soc., B.*, **178**, 417.

HOWARD, J. G., CHRISTIE, G. H. and COURTENAY, B. M. (1972). 'Studies on immunological paralysis. VII. Rapid reversal of Felton's paralysis as evidence for "tolerant cells".' *Proc. roy. Soc., B.*, **180**, 347.

HOWARD, J. G., CHRISTIE, G. H., COURTENAY, B. M., LEUCHARS, E. and DAVIES, A. J. S. (1971b). 'Studies on immunological paralysis. VI. Thymic-independence of tolerance and immunity to type III pneumococcal polysaccharide.' *Cell. Immunol.*, **2**, 614.

HOWARD, J. G., CHRISTIE, G. H., JACOB, J. and ELSON, J. (1970). 'Studies on immunological paralysis. III. Recirculation and antibody-neutralizing activity of $^{14}$C-labelled type III pneumococcal polysaccharide in paralysed mice.' *Clin. exp. Immunol.*, **7**, 583.

HUMPHREY, J. H., PARROT, D. M. V. and EAST, J. (1964). 'Studies on globulin and antibody production in mice thymectomized at birth.' *Immunology*, **7**, 419.

JERNE, N. K., NORDIN, A. A. and HENRY, C. (1963). 'The agar plaque technique for recognizing antibody-producing cells.' *Cell-Bound Antibodies* (Ed. by B. Amos and H. Koprowski), p. 109. Wistar Institute Press.

KABAT, E. A. and MAYER, M. M. (1961). *Experimental Immunochemistry*. 2nd Edition, pp. 7 and 530. Charles C. Thomas, Springfield, Illinois.

MILLER, J. F. A. P. and MITCHELL, G. F. (1968). 'Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes.' *J. exp. Med.*, **128**, 821.

MIRANDA, J. J., ZOLA, H. and HOWARD, J. G. (1972). 'Studies on immunological paralysis. X. Cellular characteristics of the induction and loss of tolerance to levan (polyfructose).' *Immunology*, **23**, 843.

MITCHISON, N. A. (1971). 'Cell cooperation in the immune response. The hypothesis of an antigen presentation mechanism.' *VIth International Immunopathology Symposium* (Ed. by P. Miescher). p. 52.

MÖLLER, G. (1970). 'Immunocyte triggering.' *Cell. Immunol.*, **1**, 573.

NOSSAL, G. J. V., AUSTIN, C. M. and ADA, G. L. (1965). 'Antigens in immunity. VII. Analysis of immunological memory.' *Immunology*, **9**, 333.

PLAYFAIR, J. H. L. and PURVES, E. C. (1971). 'Antibody formation by bone marrow cells in irradiated mice. I. Thymus-dependent and thymus-independent responses to sheep erythrocytes.' *Immunology*, **21**, 113.

RAFF, M. C. (1970). 'Role of thymus-derived lymphocyte in the secondary humoral immune response in mice.' *Nature (Lond.)*, **226**, 1257.

SERCARZ, E. and BYERS, V. S. (1967). 'The X-Y-Z scheme of immunocyte maturation. III. Early IgM memory and the nature of the memory cell.' *J. Immunol.*, **98**, 836.

SISKIND, G. W. and HOWARD, J. G. (1966). 'Studies on the induction of immunological unresponsiveness to pneumococcal polysaccharide in mice.' *J. exp. Med.*, **124**, 417.

TAYLOR, R. B. and WORTIS, H. H. (1968). 'Thymus dependence of antibody response: variation with dose of antigen and class of antibody.' *Nature (Lond.)*, **220**, 927.

UNANUE, E. R. (1970). 'Thymus dependency of the immune response to haemocyanin. An evaluation of the role of macrophages in thymectomized mice.' *J. Immunol.*, **105**, 1339.