Immunology, 1969, **16**, 527.

Regulation of Antibody Synthesis Against *Escherichia coli* Endotoxin

III. INDUCTION OF IMMUNOLOGICAL PARALYSIS IN NON-IMMUNE AND PRE-IMMUNIZED MICE

SVEN BRITTON*

Department of Tumour Biology, and Department of Bacteriology, Karolinska Institute Medical School, Stockholm 60, *Sweden*

(*Received* 12*th September* 1968)

Summary. Normal mice injected with a paralysing dose of endotoxin are unresponsive to an immediate subsequent injection of an immunogenic dose of the corresponding bacteria.

In pre-sensitized mice the injection of a paralysing dose of endotoxin suppresses the immune response after a lag period of 80–90 hours during which the responding cells appear normally. The suggested explanation to this was that antigen-sensitive cells once triggered by the antigen divide and produce antibodies for a certain time period and thereafter disappear. During this period they are unaffected by the paralysing dose of antigen. It is suggested that the cells amenable to suppression are those from which the antibody producing cells are recruited, e.g. the antigen sensitive cells.

The kinetics of suppression of an active immune response was the same whether antiserum or a paralysing dose of antigen was used as suppressive agent. This finding further supported the conclusion that actively antibody producing cells are antigen independent.

INTRODUCTION

Specific immunological unresponsiveness represents a central failure of the immunological system, the cells affected being the specifically competent lymphocytes, as judged by the inability of lymphocytes derived from paralysed animals to restore the specific immunological capacity of irradiated secondary hosts. Expression of paralysis against non-reproducing antigens is limited in time, recovery of immunological competence probably being dependent on the appearance of new immunocompetent clones in the absence of paralysing levels of antigens (Dresser and Mitchison, 1968).

The mechanism of induction of paralysis at the cell level is unknown. The simplest possibility is that the paralytogen acts by killing the competent cell (Talmage, 1957). Alternatively, paralysis is caused by non-lethal inactivation of the competent lymphocytes. Although it has been claimed that paralysis in certain antigenic systems does not show the same strict specificity as immunity (Liacopoulous and Goude, 1964; Austin and Nossal, 1966) it seems plausible that the binding between the paralytogen and the competent lymphocyte is effected by an antigen-specific receptor possibly of γ-globulin nature on the

* Present address: National Institute for Medical Research, Mill Hill, London, N.W.7.

competent lymphocyte (Dresser and Mitchison, 1968). Indirect evidence suggest that this receptor has the same specificity and physical characteristics as the γ-globulins which are produced by the cell itself or its progeny (Cross and Mäkelä, 1968).

Several investigators have observed a lag period before expression of paralysis. The duration of antigen exposure necessary for the expression of paralysis in different test systems has varied between 2 hours (Mitchison, 1968) and one (Simonsen, 1962) or several days (Golub and Weigle, 1967). The existence of a lag period preceding expression of paralysis in susceptible cells suggests that the paralytogen does not exert an immediate cytotoxic effect on the responsive cells.

This report is concerned with the kinetics of induction of paralysis against a detoxified lipopolysaccharide of *E. coli* 055:B5 in non-sensitized and pre-sensitized adult mice studied at cellular and serum level.

MATERIALS AND METHODS

Animals

Inbred male CBA mice aged 2 months were used throughout the experiments.

Antigens

Lipopolysaccharide of *E. coli* 055:B5 origin was prepared and detoxified as described before (Britton, 1969). Heat killed *E. coli* 055:B5 were suspended in saline at a concentration of 750×10^6 organisms/ml. Sheep red blood cells (SRBC), kept in Alsever's solution, were used for immunization within 1 week after bleeding. SRBC used as targets in the agar plaque test were 7–21 days old.

Immunization

All antigens were injected in one of the lateral tail veins. The immunizing dose of *E. coli* was 150×10^6 organisms suspended in 0·2 ml saline. Four times 10^8 SRBC suspended in 0·25 ml saline was used to immunize against this antigen.

The agar plaque technique

This technique for detection of single antibody forming cells against SRBC and *E. coli* endotoxin was performed as described before, using SRBC or SRRC sensitized with endotoxin, respectively, as targets *in vitro* (Britton, 1969). Only direct (19S) plaque-forming cells (PFC) were investigated.

Serology

Haemolysins and agglutinins against SRBC and *E. coli* endotoxin, respectively, were detected and quantitated as described previously (Britton, 1969). Treatment of antisera with 2-mercaptoethanol (ME) was performed as described by Uhr and Finkelstein (1963).

Passive transfer of antibodies

Pooled antiserum (haemolytic titre 1 : 2048) from mice hyperimmunized against heat killed *E. coli* was injected intravenously (0·2 ml) into mice, which had received heat killed *E. coli* 20 hours previously.

RESULTS

KINETICS OF INDUCTION OF PARALYSIS AGAINST ENDOTOXIN IN NORMAL MICE

Normal mice were injected with a paralysing (8 mg) and a sensitizing (0·8 mg) dose, respectively, of endotoxin and at various times (1–72 hours) later they were challenged with an optimal immunizing dose of the corresponding *E. coli* bacteria. The immune response was evaluated 4 days after bacterial immunization. The aim of the experiment was to investigate whether an immunogenic dose of bacteria could stimulate an immune response in animals that had been shortly before injected with a paralysing dose of endotoxin. It had been shown previously (Britton, 1969) that animals exposed to a paralysing dose of endotoxin do not produce antibodies when challenged with the corresponding bacteria 8–21 days later.

Regardless of the time interval between the injection of endotoxin and bacteria, the mice given 8 mg endotoxin were unresponsive to the subsequent injection with bacteria (Fig. 1). Mice pre-treated with 0·8 mg endotoxin always produced more 19S PFC than animals given bacteria alone (Fig. 1). The serum titres paralleled the PFC response except that sera from mice injected with 8 mg endotoxin did not contain detectable antibodies.

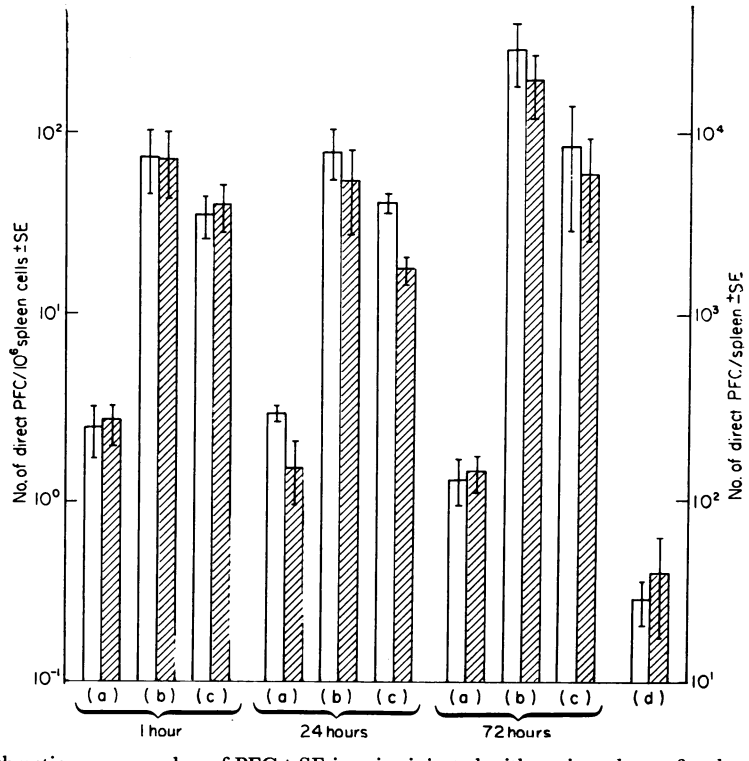

FIG. 1. Arithmetic mean number of PFC ± SE in mice injected with various doses of endotoxin and/or bacterial vaccine. Number of PFC determined 4 days after bacterial immunization. Open columns, PFC/spleen; hatched columns, PFC/10^6 spleen cells. Five mice per group. (a) 8 mg endotoxin; (b) 0·8 mg endotoxin; (c) bacteria alone; (d) no treatment.

INDUCTION OF PARALYSIS IN IMMUNIZED MICE

Experiments were performed to study the kinetics of induction of paralysis in immunized mice. In a first experiment different groups of animals were injected with bacteria 5, 4, 3, 2 and 1 days, respectively, before the test day. Three days after administration of the bacteria the animals were given 10 mg endotoxin. The time interval between endotoxin injection and the test day represented the period during which the paralysing dose of endotoxin was allowed to act in the immunized animal. Consequently, these time periods were 48 hours (in the animals injected with bacteria 5 days before the test), 24 hours (bacteria 4 days before test) and 6 hours (bacteria 3 days before test). The endotoxin-treated groups were compared with groups treated with bacteria alone. As seen in Fig. 2, all endotoxin-treated animals displayed an enhanced number of PFC, whereas their serum titres were below the detectable threshold. Evidently, paralysis was not expressed within 48 hours after the injection of the paralytogen in immunized mice. Therefore, it was tried to increase the time period of exposure to a paralysing antigen dose in the immunized animals to 144 hours. For this purpose 10 mg endotoxin was injected 24 hours after

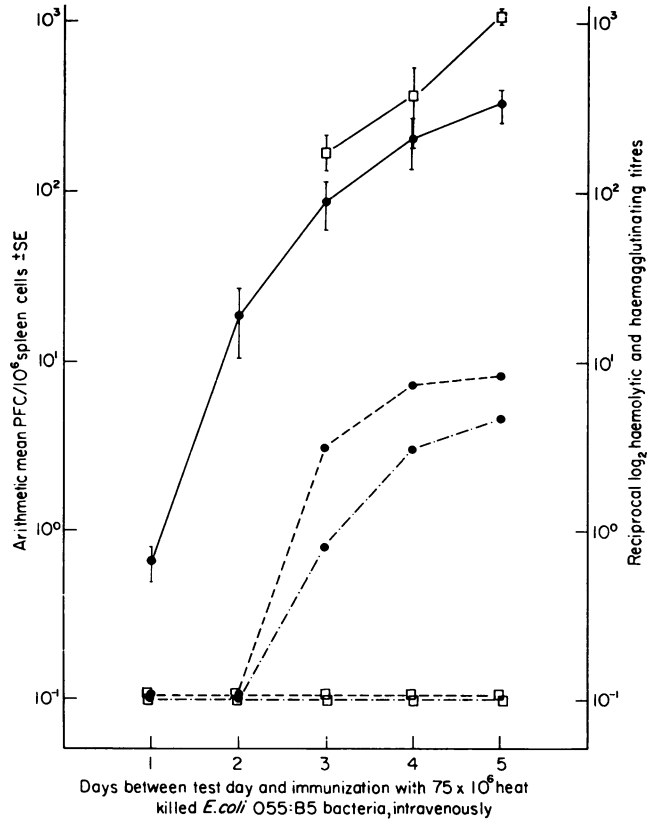

FIG. 2. Arithmetic mean number of PFC/10^6 spleen cells ±SE in mice injected with bacterial vaccine (●—— ●) and bacterial vaccine plus 10 mg endotoxin at varying time thereafter (□—— □) (see text). ● - - - - ●, Mean $\log_2$ haemolytic titres; ● - · - · - ●, mean $\log_2$ haemagglutination titres in animals injected with bacteria alone; □ - · - · - □, □ - - - - □, serum titres of animals injected with bacteria plus endotoxin.

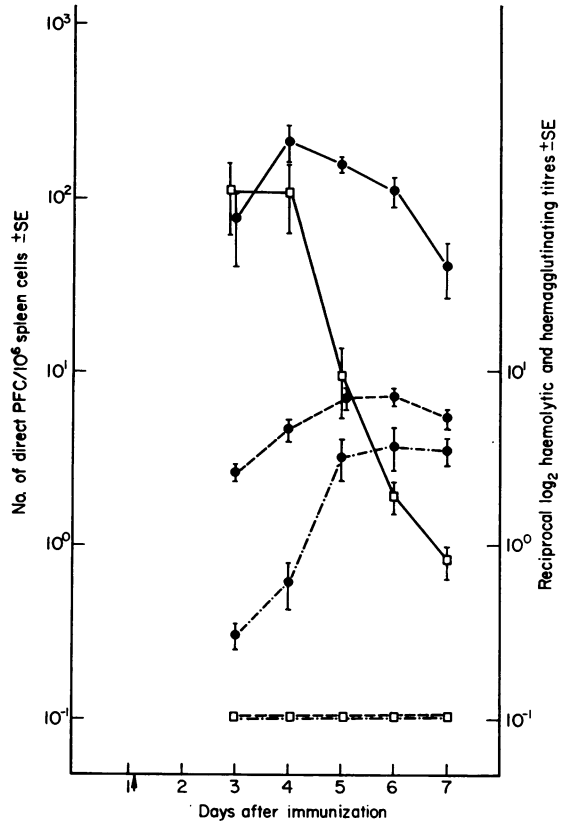

FIG. 3. Arithmetic mean number of PFC/10^6 spleen cells ±SE in animals injected with bacterial vaccine alone (●——●) or bacterial vaccine plus 10 mg endotoxin at time indicated by arrow (□——□). ● – – –●, Mean $\log^2$ titres ± SE in haemolysis, and haemagglutination (● – · – · – ●) of animals injected with bacterial vaccine. □ – – –□, □ – · – · – □, serum titres of animals injected with bacteria plus endotoxin. Five mice per group and test day.

immunization with bacteria and the degree of antibody synthesis was followed at both cellular and serum levels from 48 hours after endotoxin injection. The number of 19S PFC in the spleens of animals challenged with endotoxin was compared to that of animals injected with bacteria alone.

It was found (Fig. 3) that animals injected with endotoxin 48 hours before the test day were comparable to controls given bacteria only, with regard to their number of 19S PFC, whereas those injected with endotoxin 72, 96, 120 or 144 hours before the test day exhibited a reduction of the number of PFC, the degree of suppression increasing markedly with the time of exposure to endotoxin. No serum antibody titres could be detected in the endotoxin treated groups throughout the test period, whereas the controls immunized with bacteria developed serum antibodies as expected.

As a control for the specificity of suppression, endotoxin was administered as in the experiments shown in Fig. 3, but the mice were previously immunized with SRBC instead of *E. coli*. As can be seen from Fig. 4, parallel cellular and humoral antibody formation against SRBC occurred in both endotoxin treated and control groups, although a higher number of PFC was found in the endotoxin injected groups. Serum titres of ME sensitive

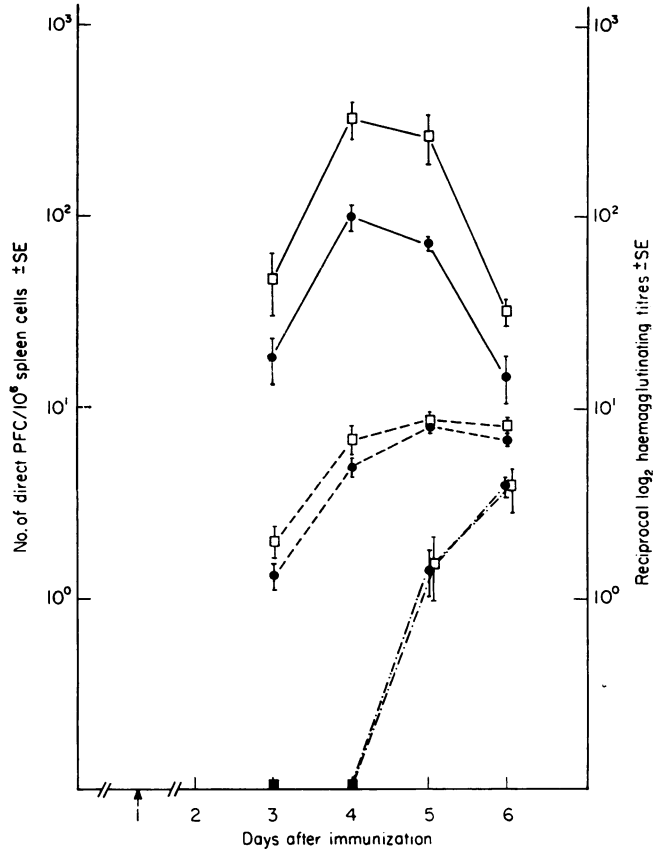

FIG. 4. Arithmetic mean number of PFC/10^6 spleen cells ±SE in mice immunized with 4×10^8 SRBC intravenously (●—— ●) and mice in addition injected with 10 mg endotoxin at time indicated by arrow (□—— □). Mean $\log_2$ titres in haemagglutination ±SE before (● - - - ●, □ - - - □) and after (● - . - . - ●, □ - . - . - □) 2-ME treatment.

and resistant antibodies appeared to be identical in the two groups. This experiment indicates that the paralysing effect of 10 mg endotoxin is immunologically specific.

From the above findings it seems likely that antibody synthesizing cells are not immediately inactivated and/or killed by the paralytogen. Attempts were made to study the time needed for an antigen to trigger antigen-sensitive cells into antibody synthesizing cells. An immunizing dose of bacteria was injected and 1, 6 or 24 hours after the animals were treated with 10 mg of endotoxin. The number of PFC was recorded 4 days after immunization and the endotoxin treated groups were compared to groups injected with bacteria alone. This experiment (Fig. 5) showed that animals immunized with bacteria and 1 hour later challenged with endotoxin were suppressed, whereas those challenged 6 and 24 hours after immunization exhibited an immune response which was close to that of controls given bacteria alone. This indicates that 24 hours is enough for the antigen to trigger competent cells into antibody secreting cells and that the triggered cells are unaffected by a paralysing load of antigen for a certain time period at least, perhaps because they already produce enough antibody to neutralize the antigen before the latter inactivates and/or kills the cells.

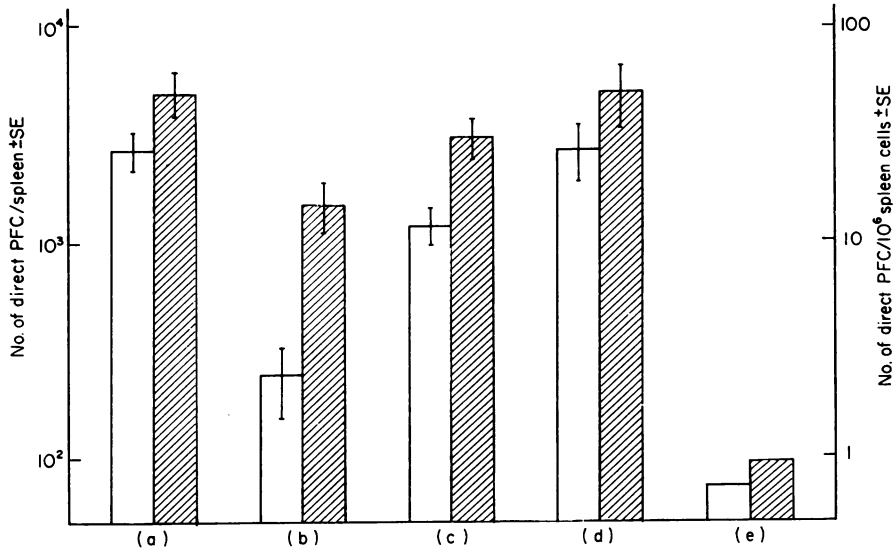

FIG. 5. Arithmetic mean number of PFC per spleen (open columns) or per 10^6 spleen cells (hatched columns) in mice 3 days after injection with bacteria intravenously or, in addition, 10 mg endotoxin at varying time thereafter. (a) Bacteria alone; (b) bacteria at time 0 + 10 mg endotoxin after 1 hour; (c) as (b) after 6 hours; (d) as (b) after 24 hours; (e) no treatment.

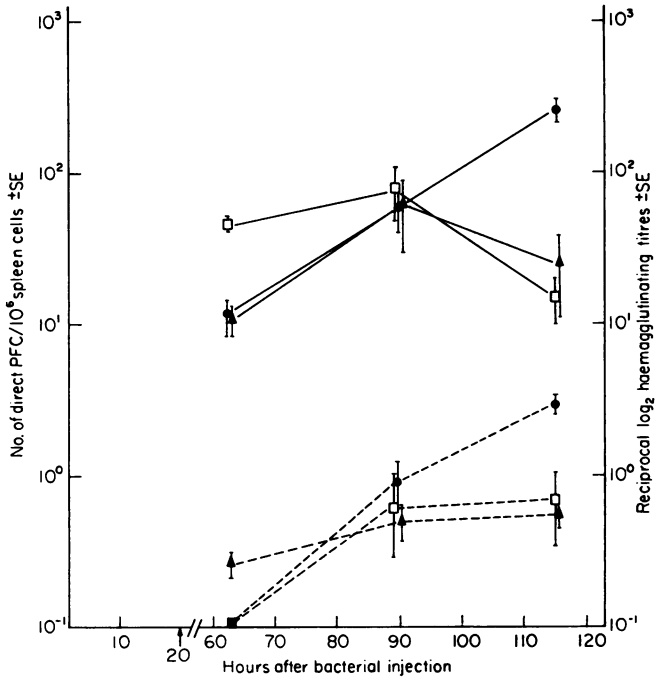

FIG. 6. Arithmetic mean number of PFC/10^6 spleen cells ± SE of mice injected with bacteria (●——●), bacteria plus antiserum (▲——▲) or bacteria plus 10 mg endotoxin (□——□) at time indicated by arrow. The same symbols with broken lines indicates mean $\log_2$ titres in haemolysis ± SE.

COMPARISON BETWEEN THE KINETICS OF SUPPRESSION BY ANTIBODY AND PARALYTOGEN

When mice are immunized with SRBC and 1–3 days later injected with anti-SRBC serum, about 40–50 hours passes before the suppressive action of the antiserum is expressed at the level of the 19S producing cells (Möller and Wigzell, 1965). This has been explained by assuming that antigen sensitive cells that have already been triggered by antigen at the time of injection of antiserum, have an intrinsic capacity to divide for 40–50 hours in the absence of effective antigenic stimuli, and thereafter disappear, unless further antigenic stimulation is provided.

Since a lag period of similar order of magnitude was found with regard to the suppressive effect of a paralysing dose of antigen on immunized animals in the present system, the kinetics of suppression of an active immune response by antigen and antibody, respectively, was compared in the same experiment.

Eight milligrams of endotoxin and 0·2 ml of anti-*E. coli* 055:B5 antiserum were injected into mice that had been immunized 20 hours earlier with an immunogenic dose of *E. coli* bacteria. The cellular and humoral immune response of these animals was followed and compared with that of animals injected with bacteria alone. The kinetics of suppression was the same whether antiserum or a paralysing dose of antigen was given and suppression was evident only after a lag phase of approximately 90 hours (Fig. 6).

DISCUSSION

It has been established in a preceding paper (Britton, 1969) that adult mice can be specifically paralysed by a high dose of detoxified endotoxin. These experiments demonstrated that induction of paralysis in non-sensitized mice was faster than induction of immunity. However, in sensitized animals a distinct lag period precedes the expression of paralysis at the cell level.

There are certain possibilities of misinterpretation inherent in studies utilizing endotoxin antigens. Thus, lipopolysaccharides from Gram-negative bacteria are toxic substances, even when they have been subjected to a detoxifying treatment. It is known that many substances are immunosuppressive when administered in doses close to the LD50 range (Makinodan, Albright, Perkins and Nettesheim, 1965), although this does not seem to be equally valid for substances that stimulate immune responses non-specifically (Hanna and Watson, 1968). It was essential, therefore, to establish that the suppressive effects of high doses of endotoxins found in the experiments reported here were not caused by non-specific toxicity. Since the injection of 10 mg endotoxin did not suppress an active immune response to SRBC (Fig. 4) or abolish the development of immunity to a subsequent injection of SRBC (Britton, 1969), it seems likely that the induction of specific immunological paralysis against bacterial lipopolysaccharides was the most likely explanation.

Normal mice injected with a paralysing dose of endotoxin do not exhibit any detectable immune response to a subsequent injection of an immunogenic dose of the homologous bacteria, even if the time intervals between the injections are very short (1 hour) (Fig. 1). This is in agreement with the finding of Golub and Weigle (1967) who observed that a simultaneous injection of a paralysing dose of soluble human γ-globulin and the same antigen incorporated in Freund's adjuvant resulted in unresponsiveness. Thus, the paralytogen inactivates the antigen-sensitive cells of the normal animal faster and/or more efficiently than they can be triggered into immune activation by the immunogen. The time needed for this inactivation to take place must at most be less than 3 days,

since a clearly detectable primary immune response to endotoxins can be recorded 3 days after administration of these antigens into mice.

Kinetic data for the induction of paralysis in the pre-sensitized animal were obtained in the present experiments. When an animal had been injected with an immunizing dose of bacteria and was challenged 24 hours later with a paralysing dose of endotoxin, it displayed a normal exponential increase of the number of 19S PFC for at least 60 hours, before suppression was expressed at the cell level. Thereafter, the decay of antibody producing cells was rapid compared to the decline after the peak in animals inmunized with bacteria alone. Even though the cellular immune response was unaffected in animals challenged with a high dose of endotoxin for 60 hours no humoral antibodies could be demonstrated. It seems probable that the serum antibodies were absorbed by the large amount of endotoxin, whereas antibody forming cells were unaffected by the excess of antigen due to the washing procedure employed for the preparation of the cell suspensions.

The finding that the number of antibody producing cells increased for 70–90 hours during a continuous load of large amounts of antigen, which eventually resulted in paralysis, was unexpected, but may be explained in the following way. Antigen-sensitive cells are triggered by the antigen into immune activation and cell division, but once the transformation from antigen-sensitive to antibody-producing cells has occurred, the cells are insensitive to antigen. The triggered cells divide and produce antibodies for a certain time period and thereafter disappear. Their rate of division or time of persistence would not be affected by addition of antigen as demonstrated in the present experiments, and would not be influenced by removal of antigen, as shown previously (Möller and Wigzell, 1965). Thus, the antibody-producing cells represent a differentiation pathway, unaffected by immunogenic or paralysing procedures. The cells amenable to suppression are those from which the antibody producing cells are recruited, e.g. the antigen-sensitive cells.

The plausibility of this explanation is strengthened by the finding that there is a striking similarity in the kinetics of suppression of immune response by means of passively administered antiserum and administration of a paralysing dose of antigen (Fig. 6). Antiserum-mediated suppression of the immune response is supposed to be effected by shielding of stimulating antigenic determinants by the passively administered antibodies. Although the interaction between the stimulating antigenic determinants and the transferred antibodies can be supposed to take place very rapidly, the immune response will proceed normally at the cell level for a certain time before suppression is expressed.

The similarity of the kinetics of suppression of the immune response by antiserum and by antigen does not imply that the underlying mechanisms are the same. On the contrary, passively administered antibodies deviate the antigen from the immunological machinery, whereas the paralysing antigen is supposed to inactivate the non-stimulated antigen-sensitive cell more directly. It seems likely, however, that both act at the level of immunocompetent cells, e.g. at the level of recruitment of new cells into antibody formation.

The postulate that antigen-sensitive cells that have been hit by the antigen and thereby triggered into differentiation into antibody producing cells are not immediately acted upon by a paralysing dose of antigen is further supported by the finding that animals immunized at time 0 and challenged with a paralysing dose of antigen 24 hours later, display a normal immune response 48 hours after the administration of the paralytogen (Fig. 5). This finding also hints at the time needed for the antigen to build up the cell population responsible for the exponential phase of the immune response towards endotoxins.

ACKNOWLEDGMENTS

I thank Mrs Rena Einars for expert technical assistance. This investigation was supported by the Swedish Cancer Society and the Damon Runyon Foundation (DR6-959).

REFERENCES

AUSTIN, C. M. and NOSSAL, G. J. V. (1966). 'Mechanism of induction of immunological tolerance. III. Cross tolerance amongst flagellar antigens.' *Aust. J. exp. Biol. med. Sci.*, **44**, 341.

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

CROSS, A. M. and MÄKELÄ, O. (1968). 'Selective inhibition of the secondary response of primed cells by incubation with hapten'. *Immunology*, **15**, 389.

DRESSER, D. W. (1965). 'Specific inhibition of antibody production. IV. Standardization of the antigen elimination test: immunological paralysis of mice previously immunized.' *Immunology*, **9**, 261.

DRESSER, D. W. and MITCHISON, N. A. (1968). 'The mechanism of immunological paralysis.' *Advanc. Immunol.*, **8**, 129.

GOLUB, E. S. and WEIGLE, W. D. (1967). 'Studies on immunological unresponsiveness. II. Kinetics.' *J. Immunol.*, **99**, 624.

HANNA, E. E. and WATSON, D. W. (1968). 'Host-parasite relationships among group A streptococci. IV. Suppression of antibody response by streptococcal pyrogenic exotoxin.' *J. Bact.*, **95**, 14.

LIACOPOULOS, P. and GOODE, J. H. (1964). 'Transplantation tolerance induced in adult mice by protein overloading of donors.' *Science*, **146**, 1305.

MAKINODAN, T., ALBRIGHT, J. F., PERKINS, E. H. and NETTESHEIM, B. (1965). 'Suppression of immunological responses.' *Med. clin. N. Amer.*, **49**, 1569.

MITCHISON, N. A. (1968). 'Immunological paralysis induced by brief exposure of cells to protein antigens.' *Immunology*, **15**, 531.

MÖLLER, G. and WIGZELL, H. (1965). 'Antibody synthesis at the cellular level. Antibody-induced suppression of 19S and 7S antibody response.' *J. exp. Med.*, **121**, 969.

SIMONSEN, M. (1962). 'Graft versus host reactions. Their natural history and applicability as tools of research.' *Progr. Allergy*, **6**, 349.

TALMAGE, D. W. (1957). 'Allergy and immunology.' *Ann. Rev. Med.*, **8**, 239.

UHR, J. W. and FINKELSTEIN, M. S. (1963). 'Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ΦX174.' *J. exp. Med.*, **117**, 457.