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Suppression of the primary immune response *in vivo* to sheep red blood cells by B-cell mitogens

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Summary. Polyacrylic acid (PAAC), lipopolysaccharide (LPS), dextran sulphate (DS) and purified protein derivative of tubercle bacilli (PPD), compounds mitogenic for B lymphocytes *in vitro*, suppressed the immune response of mice to SRBC *in vivo*, when injected 4–2 days before immunization. The same compounds enhanced the immune response when injected half an hour before immunization of the animals with a suboptimal antigen dose. A subsequent injection of PAAC given shortly before immunization, abolished the immunosuppressive effect expected by pretreatment of the animals with either PAAC or LPS. A second injection of LPS abolished the immunosuppressive effect of LPS only.

The results indicate that when B lymphocytes react with a mitogen in the absence of a particular antigen, they temporarily lose their capacity to respond to antigen.

INTRODUCTION

The adjuvant activity *in vivo* of certain compounds such as DS, PPD and LPS, has been associated with their mitogenic effect on B cells *in vitro* (Diamantstein, Rühl, Vogt and Bochert, 1973; Sultzer and Nilsson, 1972; Andersson, Sjöberg and Möller, 1972). If activation of B cells occurs also *in vivo*, it is probably a transient event, because the potentiation of the immune response by the above named compounds is dependent on the time elapsed between their injection and the antigen administration (Diamantstein, Wagner, Beyse, Odenwald and Schulz, 1971b). It is thus possible, that if a longer period of time is allowed to elapse between the injection of a B-cell mitogenic adjuvant and the antigen administration, subsequent changes will occur in the B-cell population. These changes may result either in lack of potentiation of the immune response or even in immunosuppression. Thus, it has been shown in an analogous way, that a T-cell mitogen, Con A, suppressed the immune response to SRBC when given 2 days prior to immunization (Egan, Reeder and Ekstedt, 1974). Interaction of Con A with T cells was held responsible for this effect (Egan *et al.*, 1974). Similarly, pretreatment with a bacterial endotoxin suppressed the immune response to SRBC, but this effect was related to the toxicity of the lipopolysaccharide used (Franzl and McMaster, 1968).

The aim of the present work was to find out whether prior administration of certain B-cell mitogens at a 'non-optimal time' would have a suppressive effect on the primary immune response *in vivo* to SRBC. For this purpose, B-cell mitogens known to

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enhance the immune response when given shortly before immunization were chosen.

MATERIALS AND METHODS

Animals

Random bred female NMRI/HAN mice, 8–10 weeks old, were obtained from the Zentralinstitut für Versuchstierzucht, Hannover, Germany.

Antigen

Sheep red blood cells (Behringwerke, Marburg) were used. The sheep erythrocytes (SRBC) were suspended in isotonic saline after washing three times in the same solution. The concentration was adjusted by counting an aliquot in a haemocytometer.

B-cell mitogens

The following compounds were tested: PPD (purified protein derivative of BCG tubercle bacilli, State Serum Institute, Copenhagen); LPS (*E. coli* 055-B5 lipopolysaccharide, Difco); DS (dextran sulphate, molecular weight 5×10^5, Serva, Heidelberg); PAAC (polyacrylic acid, molecular weight 20,000–30,000, kindly supplied by Dr Gergs, Organisch-Chemisches Institut der Freien Universität Berlin). LPS was boiled for 1 h before use.

Injections

Groups of six mice each, were injected intraperitoneally (i.p.) with 2×10^8 SRBC, if not otherwise stated. A constant single dose of the B-cell mitogen (3 mg PPD, 1 mg DS or PAAC and 0·25 mg of LPS), was injected i.p. in 0·5 ml PBS, a certain time prior to immunization, i.e. on either days 4, 3, 2, or 30 min before the SRBC injection. For comparison, the adjuvant effect of the various compounds was tested under known optimal conditions, i.e. i.p. injection 30 min before immunization with 2×10^6 SRBC/0·5 ml i.p.

Assay for plaque-forming cells (PFC)

Spleen cell suspensions were prepared in phosphate-buffered MEM medium (Diamantstein *et al.*, 1971b). For detection of direct (19S) PFC, 0·5 ml of appropriate dilutions of spleen cell suspensions were mixed with an equal volume of washed SRBC (1×10^9/ml), and with 0·1 ml of guinea-pig complement (Behringwerke, Marburg). For detection of indirect PFC 0·05 ml rabbit anti-IgG serum (Behringwerke, Marburg), diluted 1:10 with MEM was added to the mixture containing spleen cells, SRBC and complement. One hundred microlitres of the suspension mixture were placed into Cunningham chambers (Cunningham and Szenberg, 1968) and incubated for 1 h at 37°. The number of PFC per spleen was calculated by multiplication of the arithmetic means of triplicate determinations (s.e. less than $\pm 6\%$) with the dilution factors. The values obtained in indirect PFC tests (19S + 7S), were corrected for the inhibitory effect of the anti-IgG serum on formation of 19S plaques as described earlier (Diamantstein and Blitstein-Willinger, 1974).

Antibody determinations

Haemolysin titres (19S and 7S) and 7S (2-ME-resistant), were determined in individual sera as previously described (Sabet, Newlin and Friedman, 1969), starting at 1:8 serum dilution. $\log_2$ Titres are given when 1:8 = 1. Treatment with mercaptoethanol abolished haemolysis almost completely. Therefore, only titres determined in sera without ME treatment are given.

Statistical analysis

Differences in numbers of PFC and haemolysin titres between the various groups were tested for statistical significance by the Student's *t*-test (Sachs, 1968). The difference was considered significant when $2P \leqslant 0{\cdot}05$.

RESULTS

Assessment of the adjuvant effect

PPD (3 mg), DS (1 mg), PAAC (1 mg) or LPS (0·25 mg), were injected 30 min prior to immunization with 2×10^6 SRBC i.p. The 19S PFC response was determined on days 2, 3, 4 and 5 after immunization. Treatment with each one of the compounds significantly increased the number of PFC on all the days tested. The results obtained on the 4th day after immunization (optimum response), are presented in Fig. 1.

Immunosuppression of the primary immune response to SRBC *in vivo*

A single dose of PPD, LPS, DS or PAAC, was given on either the 4th, 3rd, 1st day, or 30 min before

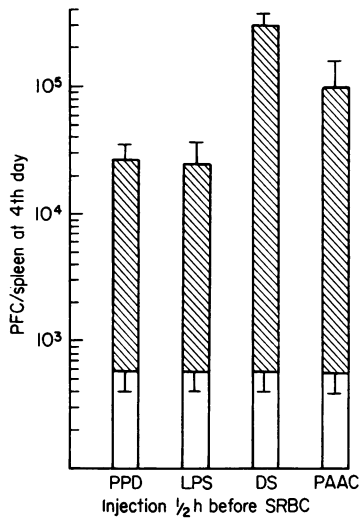

Figure 1. The effect of a single injection of B-cell mitogens on the primary immune response to 2×10^6 SRBC given i.p. The compounds PPD (3 mg), LPS (0·25 mg), DS (1 mg) or PAAC (1 mg) were injected i.p. 1/2 h before immunization. Each group consists of six animals. The PFC values represent the means of direct 19S PFC determined in individual spleens on the 4th day after immunization. The differences between the control and the treated groups were found to be significant. The standard errors are shown by the vertical bars. Control, open columns; adjuvant, hatched columns.

immunization with 2×10^8 SRBC. As shown in Fig. 2, each one of the compounds tested had an immunosuppressive effect on the response to SRBC. The maximum decrease in the PFC response was for PAAC in the groups injected on the 4th and the 3rd day, for LPS in the animals pretreated on the 3rd day before immunization and for DS or PPD in the groups injected on the 2nd day before immunization (Fig. 2a). Maximum reduction in haemolysin titres was observed in the groups pretreated with either PAAC or LPS on the 3rd day before immunization and with either PPD or DS on the 2nd day before the injection of SRBC.

The kinetics of the response to SRBC was examined in the groups pretreated with each one of the B-cell mitogens. PAAC or LPS were injected on the 3rd

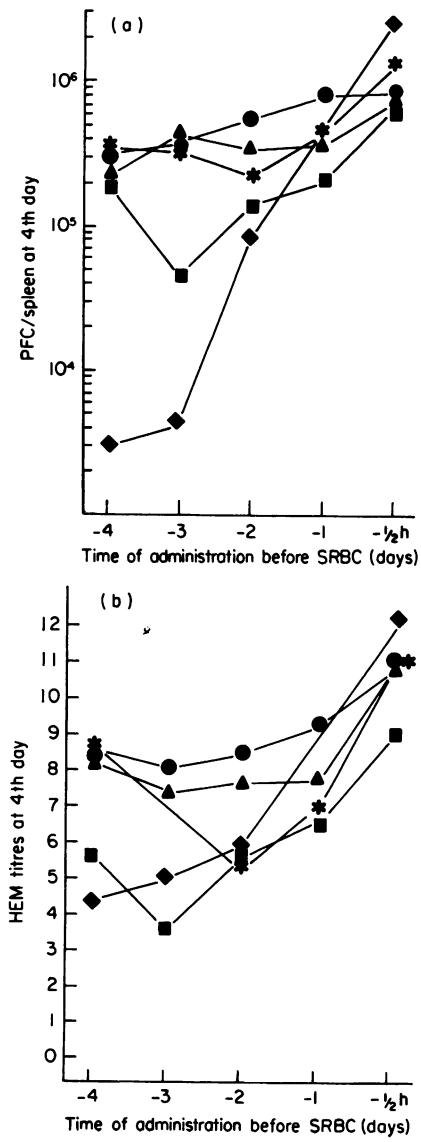

Figure 2. The effect of the time of a single injection of B-cell mitogens on the primary immune response to 2×10^8 SRBC. The compounds PPD (3 mg), LPS (0·25 mg), DS (1 mg) or PAAC (1 mg) were injected i.p. before immunization at the time indicated. Six animals per day in each group. Individual spleens and sera of mice were tested. (a) PFC. The values represent means of 19S+7S PFC as determined on the 4th day after immunization. The following differences between groups treated before immunization and untreated immunized mice were found to be significant: PPD, days 1 and 2; LPS, days 1, 2 and 3; DS, days 1 and 2; PAAC, days 2, 3 and 4 (day 1 not tested). (b) Haemolysin titres. The values represent means of individual sera taken on the 4th day after immunization. $\log_2$ Antibody titres starting at 1:8 = 1. The differences between groups treated before immunization with: PPD, days 1 and 3; LPS, days 1, 2, 3 and 4; DS days 1 and 2; PAAC, days 2, 3, and 4 (day 1 not tested), and the control groups were found to be significant. (▲) PPD; (■) LPS; (*) DS; (◆) PAAC. (●) control.

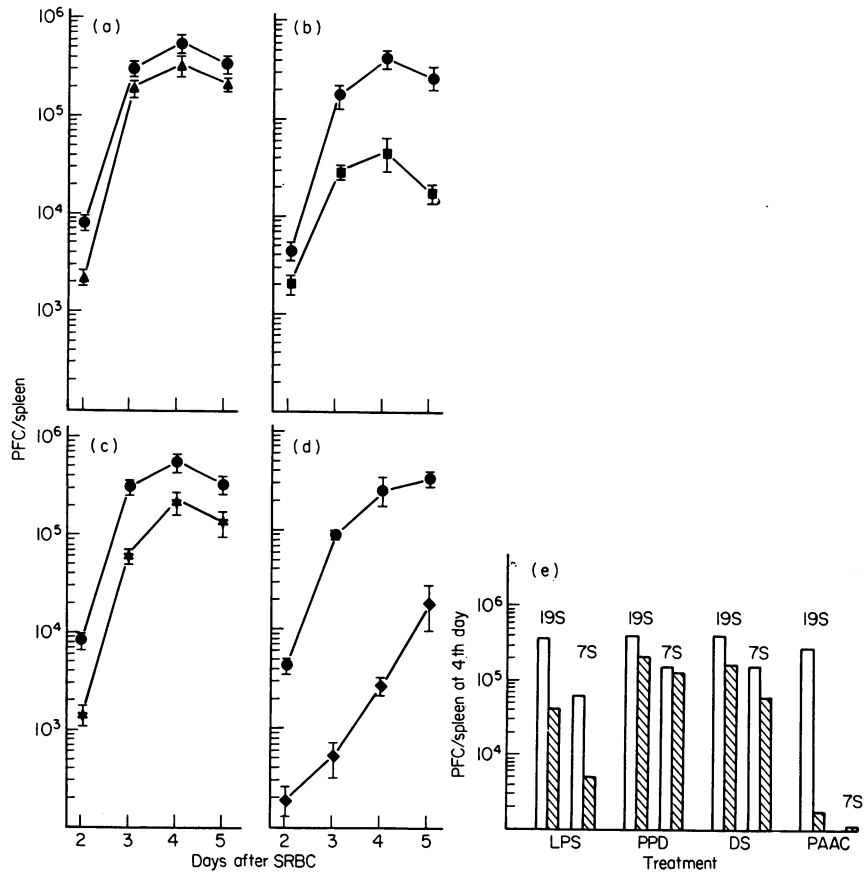

Figure 3. The effect of prior treatment with various B-cell mitogens on the kinetics of the primary immune response to 2×10^8 SRBC. The compounds were injected i.p. at the optimum time for immunosuppression. PPD and DS were injected on 2nd day and LPS or PAAC on 3rd day before immunization. The PFC values represent the mean of 19S+7S PFC detected in six individual spleens for each point. (a) Treatment with 3 mg PPD per animal. (b) Treatment with 0·25 mg LPS per animal. (c) Treatment with 1 mg DS per animal. (d) Treatment with 1 mg PAAC. The differences between the treated groups and untreated immunized groups were found to be significant on all the days tested. (e) Numbers of 19S and 7S PFC on the 4th day after immunization calculated from the data given in a, b, c and d. The standard errors in a, b, c and d are shown by the vertical bars. (●) Control groups; (▲) PPD-treated; (■) LPS-treated; (*) DS-treated; (◆) PAAC-treated; open columns, control group; hatched columns, treated groups.

day and DS or PPD were given on the 2nd day before immunization (optimum conditions for immunosuppression). The number of PFC and of haemolysin titres were determined on the 2nd, 3rd, 4th and 5th day after immunization.

The reduction in the numbers of PFC (Fig. 3.) and in haemolysin titres (Fig. 4) was evident on all the 4 days tested. As shown in Fig. 3e, both values of 19S and 7S PFC were reduced in the various pretreated groups.

Prevention of immunosuppression

The second injection of PAAC prevented almost completely the induction of immunosuppression expected from pretreatment with either PAAC or LPS. A second injection of LPS prevented the immunosuppression by LPS itself, but it had only a slight effect on the degree of immunosuppression induced by PAAC.

The results are presented in Fig. 5.

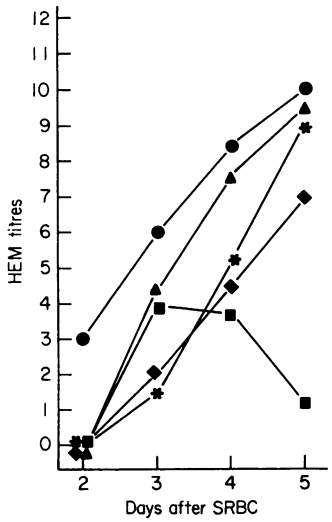

Figure 4. The effect of prior treatment with various B-cell mitogens on the kinetics of primary haemolysin response to 2×10^8 SRBC. The compounds were injected i.p. at the optimum time for immunosuppression: 2nd day for PPD and DS; 3rd day for LPS and PAAC. The haemolysin titres represent means of six individual sera per each point. $\log_2$ Titres starting from 1: 8 = 1. The differences between treated groups and controls were found to be significant on all days tested. (▲) PPD treatment; (■) LTP treatment; (*) DS treatment; (◆) PAAC treatment; (●) control.

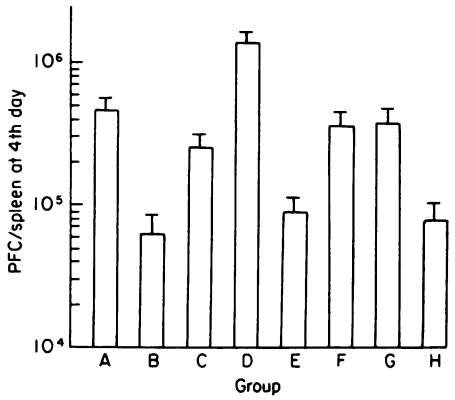

Figure 5. Abolition of the expected immunosuppressive effect by subsequent injection of either PAAC or LPS 30 min before immunization with 2×10^8 SRBC i.p. First injection of either 0·25 mg LPS or 1 mg PAAC was given on the 3rd day before immunization. The primary immune response was determined on 4th day after immunization in the following groups (six mice per group).

Group	Prior treatment
A	None
B	PAAC, 3 days
C	PAAC, 3 days and 30 min
D	PAAC, 30 min
E	LPS, 3 days
F	LPS, 3 days and 30 min
G	LPS, 3 days; PAAC, 30 min
H	PAAC, 3 days; LPS, 30 min

Mean numbers of 19S+7S per spleen—six individual determinations per group. The differences between either group B or E and each one of the groups A, C, D, F and G, were found to be significant. The standard errors are shown by the vertical bars.

DISCUSSION

The question posed in the present work is whether certain B-cell mitogens known to act as adjuvants, can have an adverse effect on the immune response if given prior to immunization at a 'non-optimal time'. The results presented here show that each one of the compounds, namely PAAC, LPS, DS or PPD can inhibit the immune response to SRBC when administered 4–2 days before immunization. The same compounds potentiated (in animals injected with a suboptimal antigen dose), or affected only marginally (in animals immunized with an optimal dose of antigen) the immune response when injected half an hour before immunization. In this respect, we confirmed previous reports on the adjuvant activity *in vivo* of PAAC (Diamantstein, Wagner, Beyse, Odenwald and Schulz, 1971a), DS (Diamantstein *et al.*, 1971b), LPS (Franzl and McMaster, 1968) and shown that PPD can also enhance the immune response *in vivo* to SRBC.

The compounds tested are mitogenic for B cells *in vitro* as shown for LPS (Andersson *et al.*, 1972), PPD (Sultzer and Nilsson, 1972), or DS (Diamantstein *et al.*, 1973) and *in vivo* as reported for PAAC (Diamantstein *et al.*, 1971a).

The adjuvant activity of B-cell mitogens has been related to their mitogenic effect (Diamantstein *et al.*, 1973; Sultzer and Nilsson, 1972; Andersson *et al.*, 1972; Watson, Trenkner and Cohn, 1973). As shown for PPD, this B-cell mitogen seems to act directly on B cells, but its adjuvant activity *in vitro* in the presence of antigen, requires also T cells and macrophages (Kreisler and Möller, 1974).

The enhancement of the immune response to SRBC by another mitogen (LPS) has been explained

by a 'two signal hypothesis'. Certain B cells receive via the receptor a signal from the antigen, but the inductive stimulus is not completed until a second mitogenic signal, which may be given by LPS, is delivered to the same cells (Watson *et al.*, 1973).

The fundamental difference in the experimental conditions leading to immunosuppression instead of potentiation, is in the time elapsed between the injection of the B-cell mitogen and of the antigen. In order to obtain an immunosuppressive effect, it is necessary to induce stimulation of cells in the absence of the test antigen, while stimulation in the presence of the antigen will either not affect the immune response or lead to enhancement. It seems, therefore, that stimulation of antigen-sensitive cells by the B-cell mitogen a few days before immunization leads to emergence of a cell population which has lost its ability to react with the corresponding antigen. The loss is a temporary one, because if the time interval between the mitogen and antigen injection is longer, the immune response is no longer depressed. Thus the immune response to SRBC was not affected when DS or PPD were injected 4 days instead of 1–3 days before the antigen injection. In the case of PAAC or LPS, injection at 14 days before immunization did not depress the immune response to SRBC (data not given here).

A certain pool of SRBC-reactive B cells is still functional even under the optimal conditions for immunosuppression. This is shown by the finding that the decrease in the immune response to SRBC is only partial and by the prevention of immunosuppression by a second injection of the B-cell mitogen, shortly before immunization. A second injection of the mitogen might stimulate again the residual pool of SRBC-reactive cells. In this respect, PAAC prevented the immunosuppression of both LPS and PAAC, while LPS was effective only towards LPS. It may be that PAAC acts on a wider spectrum of B-cell subpopulations than LPS. Such selective differences in the stimulation of B-cell subpopulations were described between DS and LPS (Diamantstein, Blitstein-Willinger and Schulz, 1974) and probably also between LPS and PPD (Andersson, Bullock and Melchers, 1974; Gronowicz, Coutinho and Möller, 1974).

The schedule of injections used by us is partly analogous to the one used in certain systems of antigenic competition against SRBC: 3–4 days time interval between the two antigens (Radovich and Talmage, 1967). Although the mechanism of antigenic competition is not yet clarified, it is possible that in some systems of competition by 'spacing' a selective effect on either B- or T-cell mitosis by the competitive antigen may be involved. In conclusion, it is suggested that the immunosuppressive effect of PAAC, DS, LPS and PPD is due to their mitogenic action on SRBC reactive B-cells in the absence of the antigen.

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