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The relation between B-cell stimulation and delayed hypersensitivity

THE EFFECT OF CYCLOPHOSPHAMIDE PRETREATMENT ON ANTIBODY PRODUCTION

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Summary. Cyclophosphamide (CY), which can enhance some forms of delayed-type hypersensitivity if given 3 days before immunization, is also a potent suppressor of most antibody mediated 4-h skin reactions to protein antigens.

However many haemagglutinating antibodies, which are present in serum at the time of skin testing, are not similarly suppressed. Antibody titres in some sera recovered from CY-pretreated guinea-pigs differ little from titres in control sera. This resistance to CY suggests that long-lived precursors characterize the B-cell lines that produce many haemagglutinating antibodies, whereas the CY-sensitive precursors of skin reactive antibodies, which mediate Arthus-type reactions, are probably rapidly dividing, short-lived cells. Furthermore, the novel appearance of BGG antibodies in sera from CY-pretreated animals immunized with DNP_{50}-BGG indicates that haemagglutinating antibody responses to some antigens are regulated by CY-sensitive mechanisms.

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INTRODUCTION

When cyclophosphamide (CY) is given to guinea-pigs 3 days before immunization with the protein antigens ovalbumin (OA) and dinitrophenyl-bovine gammaglobulin (DNP-BGG) in Freund's incomplete adjuvant (FIA), subsequent specific skin reactions are enhanced and prolonged (Turk & Parker, 1973; Scheper, Parker, Noble & Turk, 1976). In the OA experimental system, antibody production, as measured by Arthus reactivity and passive cutaneous anaphylaxis, was found to be inhibited when the delayed-type response was augmented. Increased delayed type hypersensitivity, associated with suppressed antibody response, has also been observed following CY pretreatment in experiments using mice immunized with sheep red blood cells (SRBC) and in guinea pigs with purified proteins (Lagrange, Mackaness & Miller, 1973; Willers & Sluis, 1975; Ashworth & Ford, 1976). Cyclophosphamide which eliminates rapidly dividing cells, causes preferential depletion of B-cell areas of lymph nodes and spleens (Turk & Poulter, 1972a,b). Histological evidence of B-cell depletion as well as functional evidence of B-cell nepression in CY pretreated animals exhibiting strong delayed-type hypersensitivity, has led to speculation that B cells (or their products) regulate normal delayed reactions (Katz, Parker & Turk,

1974). Thus, alteration by CY pretreatment of the appropriate balance of B and T cells could allow the expression of an exaggerated delayed-type response to many antigens.

In the present programme, we wished to observe the effect of an i.v. injection of soluble antigen just prior to immunization with antigen in adjuvant. Such a procedure had produced a persistent loss of delayed hypersensitivity and of gamma$_2$ antibody in animals immunized with bovine gammaglobulin (BGG) (Dvorak, Billote, McCarthy & Flax, 1965). However we found that the response of animals immunized with OA or DNP-substituted BGG was very different, as the i.v. injection induced strong Arthus reactivity and little evidence of suppression of delayed hypersensitivity (Scheper *et al.*, 1976). It was therefore considered important to study the effect of the i.v. injection on antibodies produced following immunization with OA, BGG or DNP-substituted BGG. All immunization protocols were repeated in animals pretreated with CY to observe parallels between increased delayed hypersensitivity reactions and alterations in B-cell function.

MATERIALS AND METHODS

Animals

Outbred Hartley strain guinea-pigs of either sex, weighing 350–500 g were used. The animals were from stocks bred at the Royal College of Surgeons or purchased from A. Tuck & Son Ltd, Rayleigh, Essex. They were fed on pelleted diet RGP (E. Dixon & Son, Ware, Herts) liberally supplemented with cabbage and hay.

Antigens

Ovalbumin (OA) five-times crystallized was purchased from Miles Seravac Ltd, Maidenhead, Berkshire.

Bovine gammaglobulin (BGG), Cohn fraction II was purchased from Sigma Chemical Co., London.

Dinitrophenyl-bovine gammaglobulin (DNP-BGG). BGG was conjugated with dinitrobenzene-sulphonic acid ($DNBSO_3$, Eastman Kodak Co.) as described by Eisen (1964). Free $DNBSO_3$ was separated from the conjugate by column chromatography on Sephadex G-25 (Pharmacia Ltd, London) and dialysis. The amount of hapten per protein molecule was calculated as described by Eisen, Carsten & Belman (1954). Two conjugates were used, one with an average of five DNP groups per molecule of BGG (DNP_5-BGG) and the other with fifty DNP groups per molecule (DNP_{50}-BGG).

Treatment with cyclophosphamide

Cyclophosphamide (CY) 'Endoxana' was kindly given by Ward Blenkinsop, Wembley, Middlesex. It was dissolved in 0·15 M NaCl and injected in a dose of 250 mg/Kg i.p.

Immunization procedures

Freund's incomplete adjuvant (FIA). The protein was dissolved in physiological saline, emulsified with an equal volume of FIA (Difco), and 0·1 ml injected into each foot pad to give a total dose of 1 μg protein per animal.

Freund's complete adjuvant (FCA). A total of 10 μg protein, emulsified in an equal volume of FCA (Difco, containing *Mycobacterium butyricum*), was injected into the foot pads of each animal, divided into four doses of 0·1 ml.

Intravenous injection of antigen. Antigens were dissolved in saline at a concentration of 5–10 mg protein/ml. A dose of 5 mg was injected i.v. into the guinea-pig ear vein, just prior to immunization.

Skin tests. Animals were skin tested by i.d. injection of 100 μg of protein in 0·1 ml of 0·15 M NaCl into the shaved flank. Reactions were assessed for increase in skin thickness using skin callipers (Schnelltäster, Kröplin AO2T). The results are expressed as specific increase in skin thickness, which represents the reading (0·1 mm) at the skin site minus the average thickness of normal skin on both sides of the skin test.

Arthus reactions were assessed 4 h after i.d. skin testing by measuring increases in skin thickness at the injection site. In this report, the Arthus reactions have been scored on an arbitrary scale in which a skin thickness increase of 0·3 mm was considered negative (−), 0·4–1·0 mm weakly positive (+), 1·1–2·0 mm strong (++) and >2·0 mm very strong (+++). All the skin reactions listed in Table 1 have been completely described and figures illustrating the time course of those reactions, as a function of increase in skin thickness, have been published elsewhere (Scheper *et al.*, 1976).

Antibody assays

Bleeding. Guinea-pigs, other than from those on

which skin tests were performed, were immunized to provide parallel serum samples. Under light ether anaesthesia, 5 ml of blood were withdrawn from each animal by cardiac puncture on day 6 and day 8 after immunization. Larger amounts of blood were taken when the animals were bled to death by cardiac puncture on day 14 or later. The blood was maintained for 1 h at 37° and for 2 h at 4°, after which serum from each sample was collected separately, inactivated for 30 min at 56°, and then stored at −20°.

Haemagglutination. Sheep red blood cells (SRBC) were coated with OA and BGG by the $CrCl_3$ method of Poston (1974). Briefly, 0·3 ml of packed, saline-washed SRBC were mixed with 0·9 ml of piperazine buffer pH 6·5, 0·9 ml of antigen (5–10 mg/ml) and 0·9 ml $CrCl_3$ (3 gm/5 ml H_2O) for 5 min at room temperature. The coating reaction was stopped by adding 10 vol. of saline and centrifuging the SRBC. After three washes in saline, protein-coated SRBC were suspended at a concentration of 1%. For testing with coated SRBC, sera to be tested were diluted serially in microtitre plates, using a 3% solution of normal guinea-pig serum in saline.

DNP-coated guinea-pig red blood cells (GPRBC) were prepared according to the method of Scheper & Oort (1974). Fresh guinea-pig blood was collected into Alsever's solution and the red cells were washed twice in saline and twice in a solution of 0·02 M Na_2HPO_4, containing 5·9 gm NaCl/litre. Thirty-drops of DNFB added to 50 ml of the Na_2HPO_4/NaCl solution provided the coating antigen. Diluted DNFB was added in a volume equal to 10 times the volume of the GPRBC pellet. Red cells and DNFB were incubated for 15 min at room temperature with occasional mixing, after which the DNP-coated red cells were centrifuged and washed twice in phosphate-buffered glucose (1·6 gms Na_2HPO_4, 0·49 gms KH_2PO_4, 5·4 gms glucose/200 ml). A 2% suspension of coated cells in the diluent was used for the microtitration.

To measure 2-mercaptoethanol (2-ME) resistant antibodies, 100 μl of inactivated antiserum was incubated with 50 μl of 0·1 M 2-ME for 1 h at 37°C. Microtitre tests were performed as usual immediately afterwards.

Mean antibody titres ± SEM, which are illustreated in Figs 1–5, were determined by including the responses of all members of a group even when individual titres were less than 1. The fraction of responders in a group is recorded below each bar in the histogram.

RESULTS

Arthus reactivity

A summary of the Arthus reactivity of animals immunized with all four antigens in FCA and FIA is given in Table 1. It can be seen that reactivity regularly occurs with OA and BGG but not with DNP-substituted BGG unless preceded by an i.v. injection of antigen. CY inhibits Arthus reactivity to all antigens except DNP_5-BGG when immunization in adjuvant is preceded by an i.v. injection of antigen.

Table 1. Four-hour skin reactions*

	Antigens			
Treatment†	OA	BGG	DNP_{50}-BGG	DNP_5-BGG
FIA	+	+	−	−
CY-FIA	−	−	−	−
IV-FIA	++	−	+	+
CY-IV-FIA	−	−	−	+
FCA	++	++	−	−
CY-FCA	−	−	−	−
IV-FCA	+++	−	++	+
CY-IV-FCA	−	−	−	+

* Details of scoring are given in the Materials and Methods section.

† FIA = Animals immunized with antigen in Freund's incomplete adjuvant; FCA = animals immunized with antigen in Freund's complete adjuvant; IV = animals injected i.v. with antigen just prior to immunization with antigen in adjuvant; CY = animals given CY (250 mg/Kg) i.p. 3 days before immunization.

Haemagglutinating antibody

(i) *OA*

Although CY suppressed Arthus reactivity to this antigen, it had a different effect on antibodies measured by passive haemagglutination (Fig. 1). Antibodies induced by i.v. injection of antigen together with antigen in adjuvant were only partially reduced or not reduced at all. Although ME-resistant antibody was normally present as early as day 6 after

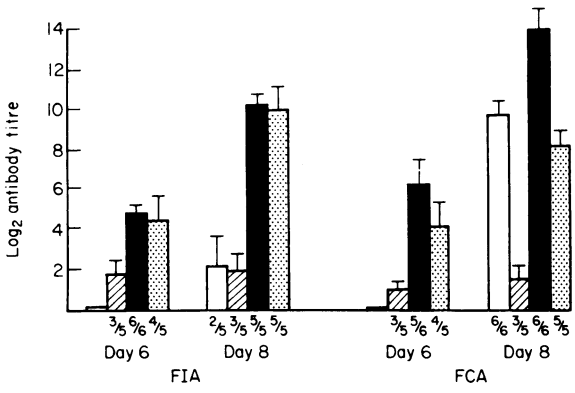

Figure 1. Passive haemagglutinating antibody titres in OA immunized guinea-pigs 6 and 8 days after immunization. The proportion of responders, when not all negative, in each group is indicated in the fractions below the bars. □ Control; ▨ CY; ■ i.v.; ⊠ CY i.v.

immunization in animals given antigen intravenously, that present in CY-pretreated groups was all ME-sensitive.

(ii) *BGG*

Pretreatment with CY, which greatly inhibited Arthus reactivity, did not suppress the synthesis of haemagglutinating antibody (Fig. 2). The i.v. injection of antigen before immunization with antigen in adjuvant significantly ($P < 0.05$) depressed antibody production. However this depression was reversed by pretreatment with CY.

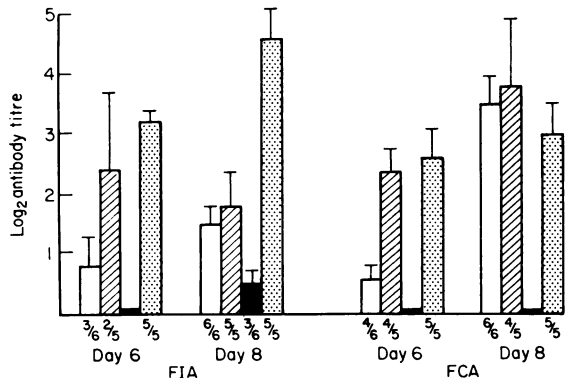

Figure 2. Passive haemagglutinating antibody titres in BGG immunized guinea-pigs 6 and 8 days after immunization. The proportion of responders, when not all negative, is indicated in the fraction below the bars. □ Control; ▨ CY; ■ i.v.; ⊠ CY i.v.

(iii) *DNP_{50}-BGG*

Sera from animals immunized with DNP_{50}-BGG were tested separately against DNP-coated guinea-pig erythrocytes (Fig. 3) and BGG-coated sheep erythrocytes (Fig. 4). Levels of DNP antibodies were unaffected by either CY pretreatment or i.v. injection of antigen before immunization with antigen in adjuvant. Antibody against BGG could not be detected as late as 14 days after immunization with or without prior treatment with i.v. antigen. In contrast, CY pretreatment resulted in the production of low titred BGG antibodies in two out of five guinea-pigs as early as 6 days after immunization with antigen in FIA and in animals given antigen i.v. the synthesis of BGG antibodies was even more efficient. All BGG antibodies produced in CY-pretreated animals were sensitive to 2-ME treatment. ME-resistant antibodies to the DNP group were only detected in animals immunized with antigen in FCA with or without i.v. antigen.

(iv) *DNP_5-BGG*

Passive haemagglutination tests on sera from animals immunized with DNP_5-BGG were similarly performed using DNP- and BGG-coated erythrocytes (Figs 5, 6). CY pretreatment had no depressive effect on DNP antibody production and markedly increased the levels found in animals pretreated with an intravenous injection of antigen. The effect of CY on BGG antibodies was the opposite to that on DNP antibodies in that the response was depressed by CY pretreatment, as well as by i.v. antigen. ME-resistant DNP antibodies were only in CY-pretreated

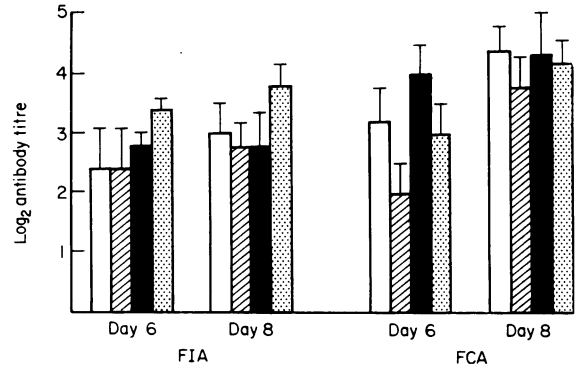

Figure 3. Passive haemagglutinating DNP antibody titres in DNP_{50}-BGG immunized guinea-pigs 6 and 8 days after immunization. All animals in every group were responders. □ Control; ▨ CY; ■ i.v.; ⊠ CY i.v.

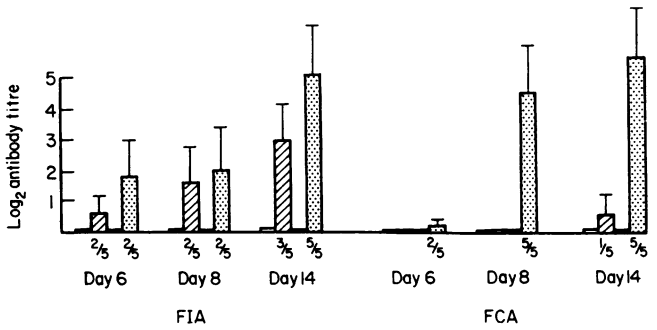

Figure 4. Passive haemagglutinating BGG antibody titres in DNP_{50}-BGG immunized guinea-pigs 6, 8 and 14 days after immunization. The proportion of responders, when not all negative, in each group is indicated in the fractions below the bars. □ Control; ▨ CY; ■ i.v.; ⊠ CY i.v.

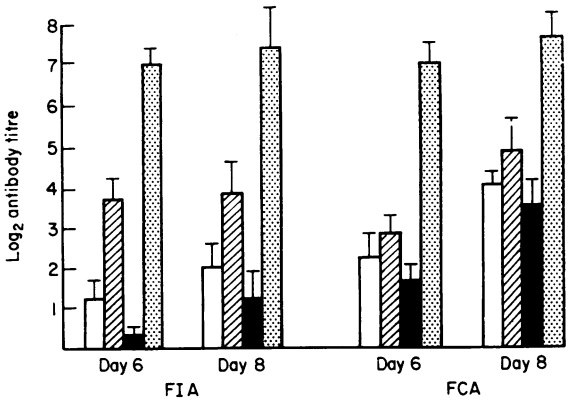

Figure 5. Passive haemagglutinating DNP antibody titres in DNP_5-BGG immunized guinea-pigs, 6 and 8 days after. immunization. All animals in every group were responders. □ Control; ▨ CY; ■ i.v.; ⊠ CY i.v.

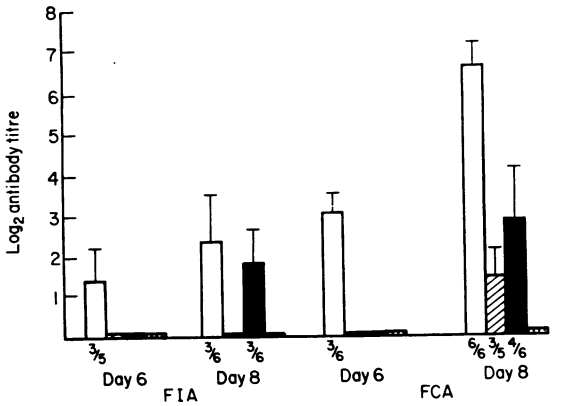

Figure 6. Passive haemagglutinating BGG antibody titres in DNP_5-BGG immunized guinea pigs 6 and 8 days after immunization. The proportion of responders, when not all negative, is indicated in the fraction below the bars. □ Control; ▨ CY; ■ i.v.; □ CY i.v.

i.v. injected animals. In both FIA and FCA immunized groups on days 6 and 8 the mean titre of ME-resistant antibody was approximately 2·0.

DISCUSSION

The data presented show that CY given in a way that increases certain delayed hypersensitivity reactions does not suppress all antibody responses to protein antigens. Although 4-h Arthus type skin reactions and the production of antibody detectable by passive cutaneous anaphylaxis are inhibited (Turk & Parker, 1973), the production of certain haemagglutinating antibodies can be enhanced. These results in the guinea-pig therefore show some agreement with those of Askenase, Hayden & Gershon (1975) who found that, in the mouse, low doses of CY induced a marked increase in delayed hypersensitivity to sheep erythrocytes without depressing the level of haemagglutinating antibody to this antigen.

The effect of i.v. injection of BGG in depressing haemagglutinating antibody production as well as the 4-h and delayed hypersensitivity reactions to BGG has been demonstrated previously by Dvorak *et al.* (1965). In previous experiments we noted that the i.v. injection of OA had the effect of increasing both immediate and delayed skin reactivity (Scheper *et al.*, 1976). This increase in immediate reactivity is paralleled by an increase in haemagglutinating antibody to this antigen. The effect of CY on haemagglutinating antibody production is also different with these two antigens. CY pretreatment allows an earlier development of antibody to BGG, whereas the initial levels of antibody to OA are not increased. However when antibody levels are high (OA/FCA

day 8) the effect is one of depression. The increased levels of antibody to OA induced by i.v. injection are not depressed in animals immunized with antigen in incomplete adjuvant, although a depression may be observed in animals immunized with complete adjuvant. The most striking effect of CY pretreatment on haemagglutinating antibody production is the reversal of the suppression of antibody production induced by the i.v. injection of antigen in the BGG model. This treatment did not however reverse the suppression of Arthus reactivity.

The response of animals immunized with minimally substituted (DNP_5) BGG to DNP and BGG is different from the response in animals immunized with the maximally substituted (DNP_{50}) antigen. In animals immunized with minimally substituted antigen, i.v. injection of antigen depresses the level of antibody to DNP if animals are immunized with incomplete adjuvant but not if complete adjuvant is used. This depression is reversed by CY pretreatment. All CY-treated, i.v. injected animals showed enhanced levels of this antibody. In contrast, the level of antibody to DNP in animals immunized with maximally substituted antigen was the same whatever immunization schedule was used.

Antibody to BGG could be detected after immunization with DNP_5-BGG in adjuvant and even in animals additionally treated with i.v. antigen. In contrast, even 14 days after immunization with DNP_{50}-BGG no antibody could be detected to the carrier. CY pretreatment however allows antibody to BGG to be made under conditions where it cannot be detected in animals not treated with this drug. The effect of CY on antibody production to BGG in minimally sensitized animals was one of depression.

To summarize, antigen injected i.v. just prior to immunization in adjuvants can either increase, decrease or have no effect on the level of haemagglutinating antibody. The effect observed would appear to depend on the nature of the antigen used. In animals immunized with BGG or BGG minimally substituted with DNP, i.v. antigen generally depresses the response. With maximally substituted BGG no effect is observed, and with OA the effect is to increase the levels. These findings are in agreement with a previous suggestion that with a weaker antigenic stimulus i.v. antigen can depress an immune response, whereas with a stronger antigen the effect may be one of enhancement (Scheper *et al.*, 1976).

The most striking effect of CY pretreatment is that in many of the systems the level of haemagglutinating antibody is greatly increased. In one system, the response to BGG in animals immunized with DNP_{50}-BGG, CY pretreatment allows the production of antibody under conditions where it would normally be undetectable. These findings are comparable to those of Polak & Turk (1974) who found a reversal of suppression of the antibody response by CY in animals made unresponsive to the DNP group by i.v. injection of dinitrobenzenesulphonate. In this system it has been suggested that unresponsiveness was due to the induction of a population of suppressor cells. It is likely therefore that a similar system operates in the control of production of haemagglutinating antibody, a control from which it is released by CY.

It is now apparent that the effect of CY on B-cell function is not as simple as has been previously suggested (Turk & Poulter, 1972b). A number of B-cell functions have been shown to be depressed by CY; these include the Arthus phenomenon to certain antigens, the production of antibodies mediating passive cutaneous anaphylaxis and the development of suppressor cells to some antigens during normal immunization (Turk, Polak & Parker, 1976). Other functions described in this paper and elsewhere (Askenase *et al.*, 1975) are not depressed. This would suggest that at least two populations of B cells exist. It could be that a high turnover population is susceptible to CY (Turk & Poulter, 1972a), whereas long-lived B cells are not affected by this treatment, and that most haemagglutinating antibodies are derived from this second population of cells.

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REFERENCES

ASHWORTH L.A.E. & FORD W.H. (1976) Mitogenic factor as an *in vitro* correlate of delayed hypersensitivity in the guinea pig. *Int. Arch. Allergy*, **50**, 583.

ASKENASE P.W., HAYDEN B.J. & GERSHON R.K. (1975) Augmentation of delayed-type hypersensitivity by doses of cyclophosphamide which do not affect antibody response. *J. exp. Med.* **141**, 697.

DVORAK H.F., BILLOTTE J.B., MCCARTHY J.S. & FLAX M.H. (1965) Immunologic unresponsiveness in the adult guinea pig. I. Suppression of delayed hypersensitivity and antibody formation to protein antigens. *J. Immunol.* **94**, 966.

EISEN H.N. (1964) Preparation of purified anti-2,4-dinitrophenyl antibodies. *Methods med. Res.* **10**, 94.

EISEN H.N., CARSTEN M.E. & BELMAN S. (1954) Studies of hypersensitivity to low molecular weight substances. III. The 2,4-dinitrophenyl groups as a determinant in the precipitation reaction. *J. Immunol.* **73**, 297.

KATZ S.I., PARKER D. & TURK J.L. (1974) B-cell suppression of delayed hypersensitivity. *Nature (Lond.)*, **251**, 550.

LEGRANGE P.H., MACKANESS G.B. & MILLER T.E. (1974) Potentiation of T-cell-mediated immunity by selective suppression of antibody formation with cyclophosphamide. *J. exp. Med.* **139**, 1529.

POLAK L. & TURK J.L. (1974) Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity. *Nature (Lond.)*, **2491** 654.

POSTON R.N. (1974) A buffered chromic chloride method of attaching antigens to red cells: use in haemagglutination. *J. immunol. Meth.* **5**, 91.

SCHEPER R.J. & OORT J. (1974) Rosette-forming cells and the immunological response after DNCB, DNP-carrier and oxazolone sensitization. *Immunology*, **261** 269.

SCHEPER R.J., PARKER D., NOBLE B. & TURK J.L. (1977) The relation of immune depression and B-cell stimulation during the development of delayed hypersensitivity to soluble antigens. *Immunology*, **32**, 265.

TURK J.L. & PARKER D. (1973) Further studies on B-lymphocyte suppression in delayed hypersensitivity, indicating a possible mechanism for Jones-Mote hypersensitivity. *Immunology*, **241** 751.

TURK J.L. & POULTER L.W. (1972a) Effects of cyclophosphamide on lymphoid tissues labelled with 5-iodo-2-deoxyuridine-^{125}I and ^{51}Cr. *Int. Arch. Allergy.* **43**, 620.

TURK J.L. & POULTER L.W. (1972b) Selective depletion of lymphoid tissue by cyclophosphamide. *Clin. exp. Immunol.* **10**, 285.

TURK J.L., POLAK L. & PARKER D. (1976) Control mechanisms in delayed-type hypersensitivity. *Brit. med. Bull.* **32**, 165.

WILLERS J.M.N. & SLUIS E. (1975) The influence of cyclophosphamide on antibody formation in the mouse. *Ann. Immunol. (Inst. Pasteur)*, **126C**, 267.