

## The effect of cyclophosphamide and role of suppressor cells in the desensitization of delayed hypersensitivity

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**Summary.** Desensitization of guinea-pigs with ovalbumin (OA) or bovine gamma globulin (BGG) induces strong specific desensitization and is associated with a non-specific anergy to tuberculin—PPD. Similarly, it was found that animals receiving desensitizing injections of PPD have suppressed delayed hypersensitivity reactions to OA or BGG. PPD also induced strong specific desensitization. Cyclophosphamide (CY) given in one large dose (300 mg/kg) 3 days before immunization failed to affect the specific desensitization induced by all three antigens. However, if CY was given 1 day after immunization, it was not possible to induce specific desensitization. The induction of non-specific desensitization was prevented in all three antigen systems if CY was given either 3 days before or 1 day after immunization. Desensitization with either OA or BGG markedly suppressed the specific 4 hr Arthus reactions.

### INTRODUCTION

Evidence suggests that delayed hypersensitivity, like most immunological reactions, appears to be positively regulated. Cyclophosphamide (CY), presumably by interfering with such mechanisms, enhances all

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forms of delayed hypersensitivity (Turk, Parker & Poulter, 1972; Turk & Parker, 1973; Dwyer, Parker & Turk, 1981). It has been suggested that the regulatory mechanisms involve a population of cells that are rapidly dividing and, therefore, susceptible to the alkylating properties of the drug.

We have previously shown that inhibition of a delayed hypersensitivity reaction to one antigen by a strong and simultaneous reaction to a second antigen (antigenic competition) is prevented by CY (Dwyer *et al.*, 1981). The anergy seen in certain human disease states, for example Hodgkins' disease and sarcoidosis, and the very similar lack of delayed hypersensitivity response in desensitized guinea-pigs, may result from similar mechanisms producing a positive reduction in T-cell effector function. We have examined the effect of CY treatment on desensitization in guinea-pigs.

Animals immunized with a soluble protein antigen in Freund's Complete Adjuvant (FCA) and given a large (mg) injection of the protein antigen 7 days after immunization become anergic (desensitization; Uhr & Pappenheimer, 1958). They fail to respond to an intradermal challenge with either the protein antigen used for desensitization (specific desensitization) or the other immunizing antigens (non-specific desensitization; Dwyer & Kantor, 1973).

In these studies we have found that CY, administered either 3 days before or 1 day after immunization, prevents the desensitizing injection from producing non-specific anergy. This inhibition of 'non-specific desensitization' is also seen when purified protein

derivative of tuberculin (PPD) is used as the desensitizing antigen and another soluble protein is the indifferent antigen. Specific desensitization is only inhibited in animals receiving CY 1 day after immunization; treatment 3 days before immunization does not alter the ability of guinea-pigs to be specifically desensitized.

## MATERIALS AND METHODS

### *Animals*

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

### *Antigens*

Ovalbumin (OA), crystallized five times, was purchased from Miles Seravac Ltd, Maidenhead, Berks. Bovine gamma globulin (BGG), Cohn fraction II, was purchased from Sigma Chemical Co., London. Purified protein derivatives of tuberculin (PPD) was given by Central Veterinary Laboratory, Weybridge.

### *Treatment with cyclophosphamide*

Cyclophosphamide (CY) 'Endoxana' was given by WB Pharmaceuticals Ltd, Bracknell, Berkshire. It was dissolved in 0.15 M NaCl and injected intraperitoneally in a dose of 300 mg/kg either 3 days before or 1 day after immunization.

### *Immunization procedures*

OA and BGG, dissolved in physiological saline, were emulsified with an equal volume of Freund's complete adjuvant (FCA, Difco, containing *Mycobacterium tuberculosis*) and 0.1 ml. was injected into each footpad. When immunizing with BGG the guinea-pigs received a total of 200 µg of antigen in 0.4 ml of emulsion, to which had been added 120 µg of ground *M. tuberculosis* (Thestrup-Pedersen, Dwyer & Askenase, 1977). However, animals immunized with OA received a total of 10 µg antigen in FCA without the added *M. tuberculosis*. This was because when using the BGG protocol with ovalbumin the PPD reactions were suppressed by the stronger antigen as a result of antigenic competition (Dwyer *et al.*, 1981). A

minimum of six animals were used in each experimental group.

### *Desensitization*

On days 7 and 8 after immunization, guinea-pigs received a subcutaneous injection of 2 mg of the desensitizing antigen in 1 ml saline.

### *Skin tests*

Animals were skin tested, immediately after desensitization on day 8, by intradermal injection of either 25 µg BGG or 100 µg OA dissolved in 0.1 ml saline. Simultaneously, they received a skin test of 25 µg PPD on the opposite flank. Reactions were assessed 4, 24 and 48 hr after skin testing by measuring the increase in skin-fold thickness and reaction size (Scheper, Noble, Parker & Turk, 1977a).

The results reported are according to the size of the reaction, which was determined by taking the average of the vertical and horizontal diameters through the centre of the reaction. The increase in skin-fold thickness paralleled the size of the reaction.

### *Statistics*

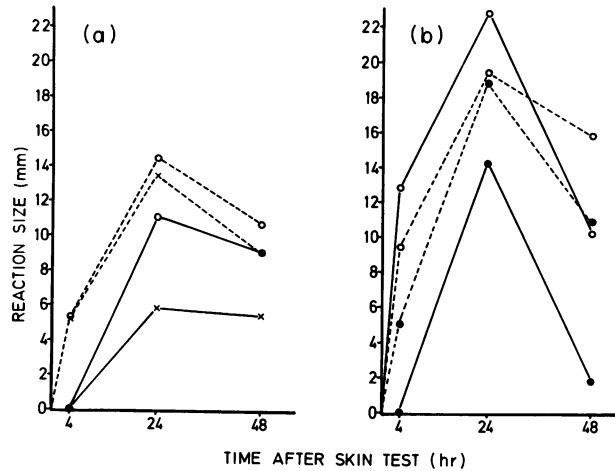
Comparisons were made between reactions in control and test animals and significant differences sought using Student's *t* test.

## RESULTS

### **Effect of CY on 'non-specific desensitization'**

*Effect of CY on desensitization with OA in guinea-pigs immunized with OA.* As previously reported CY given 1 day after immunization (CY + 1) enhanced the delayed hypersensitivity response to PPD in animals immunized with OA in FCA (Fig. 1a). In animals not treated with CY, desensitization with OA strongly suppressed the PPD skin reactions. However, in animals which had received CY 1 day after immunization, the PPD skin reactions were unaffected by the desensitizing doses of OA. In guinea-pigs which had received CY 3 days before immunization it was possible to get partial non-specific desensitization with OA, but this was not significant (Table 1).

*Effect of CY on desensitizing with PPD in guinea-pigs immunized with OA.* Subcutaneous injection of large doses of PPD into animals immunized with OA in FCA not only produced specific desensitization to PPD (Table 1), but also reduced the delayed hyper-

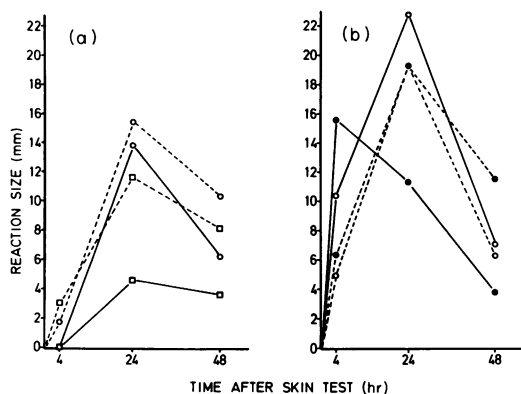


**Figure 1.** Immunization with OA. Skin test reactivity as measured by size of reaction to (a) PPD and (b) OA 8 days after immunization. All groups were immunized with OA in FCA and skin tested with OA and PPD. The notation  $\circ\text{---}\circ$  refers to the control animals skin tested with PPD in diagram (a) and skin tested with OA in diagram (b). One group received two s.c. injections of OA ( $x\text{---}x$ ), another two s.c. injections of PPD ( $\bullet\text{---}\bullet$ ). Three more groups were treated as above having had an i.p. injection of 300 mg/kg CY 1 day after immunization ( $\circ\text{---}\circ$ ,  $x\text{---}x$  and  $\bullet\text{---}\bullet$ , respectively).

**Table 1.** Effect of CY on desensitizing with either OA or PPD in animals immunized with OA in FCA

	Reaction size to PPD*			Reaction size to OA		
	4 hr	24 hr	48 hr	4 hr	24 hr	48 hr
CY +1						
Control	0	11.0 ± 3.5	9.0 ± 5.1	12.8 ± 9.4	22.9 ± 9.1	10.2 ± 8.5
CY control	5.3 ± 4.3	14.4 ± 2.1	10.6 ± 2.6	9.4 ± 2.2	19.4 ± 1.8	15.8 ± 3.2
Ds OA	0	5.8 ± 4.9	5.4 ± 3.0	2.4 ± 5.1	1.7 ± 4.1	0
CY-Ds OA	5.2 ± 3.9	13.4 ± 3.7	9.0 ± 1.8	0	16.8 ± 1.8	6.9 ± 6.7
Ds PPD	0	0	0	0	14.2 ± 11.1	1.8 ± 4.5
CY-Ds PPD	0	4.0 ± 3.8	2.5 ± 2.9	5.0	18.8 ± 1.1	10.9 ± 3.3
CY -3						
Control	0	5.6 ± 5.2	4.7 ± 4.3	12.6 ± 7.9	20.9 ± 6.9	12.0 ± 10.5
CY control	1.1 ± 3.0	10.7 ± 4.5	9.1 ± 3.5	2.1 ± 3.7	22.8 ± 12.9	10.3 ± 5.0
Ds OA	0	1.7 ± 3.0	2.2 ± 3.4	0	3.0 ± 4.9	0
CY-Ds OA	0	6.2 ± 6.2	5.7 ± 5.1	0	2.7 ± 4.4	0
Ds PPD	0	0	0	4.2 ± 6.6	10.5 ± 6.8	3.0 ± 3.5
CY-Ds PPD	0	0	0	0	21.3 ± 4.4	11.3 ± 3.0

\* Reaction size expressed as mean diameter of a minimum of six animals ± SD.



**Figure 2.** Immunization with BGG. Skin test reactivity as measured by size of reaction to (a) PPD and (b) BGG, 8 days after immunization. All groups were immunized with BGG in FCA and skin tested with BGG and PPD. The notation  $\circ$ — $\circ$  refers to the control animal skin tested with PPD in diagram (a) and with BGG in diagram (b). One group received two s.c. injections of BGG ( $\square$ — $\square$ ), another two s.c. injections of PPD ( $\bullet$ — $\bullet$ ). Three more groups were treated as above, having had an i.p. injection of 300 mg/kg CY 3 days before immunization ( $\circ$ — $\circ$ — $\circ$ ,  $\square$ — $\square$ — $\square$  and  $\bullet$ — $\bullet$ — $\bullet$ , respectively).

sensitivity reaction to OA. (Fig. 1b). However, desensitizing injections of PPD in animals given CY either 3 days before or 1 day after immunization, did not affect skin reactions to OA, in that they were the same as those in the CY-treated guinea-pigs which had not received the subcutaneous injections (Table 1).

As we have previously shown that the effect of CY on the delayed hypersensitivity reaction may vary with the antigen used (Scheper, Parker, Noble & Turk, 1977b), we repeated the above experiments using BGG.

*Effect of CY on 'non-specific desensitization' in guinea-pigs immunized with BGG in FCA.* CY administered either 3 days before or 1 day after immunization prevented desensitization of the delayed hypersensitivity reaction to PPD by BGG (Fig. 2a and Table 2). When the experiments were reversed so that PPD was used as the desensitizing antigen, it was found that, as in the OA-immunized animals, PPD was able to produce significant non-specific desensitization ( $0.01 > P > 0.001$ ). However, this was not possible in animals that had been treated with CY either 3 days before or 1 day after immunization (Fig. 2b).

These experiments show that PPD, as well as antigens such as OA and BGG, can induce 'non-specific desensitization'. However, it seems that it is not possible to induce 'non-specific desensitization' with OA, BGG or PPD if guinea-pigs have been treated with CY either 3 days before or 1 day after immunization.

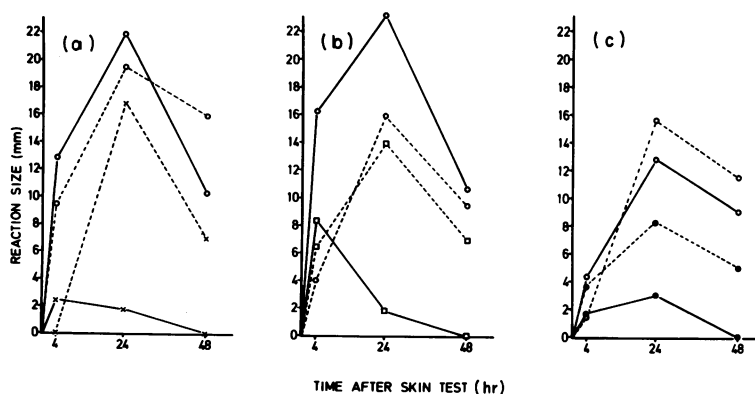
#### Effect of CY on specific desensitization

Specific desensitization occurred with all three antigens and this was significantly inhibited in animals

**Table 2.** Effect of CY on desensitizing with either BGG or PPD in animals immunized with BGG in FCA

	Reaction size to PPD*			Reaction size to BGG		
	4 hr	24 hr	48 hr	4 hr	24 hr	48 hr
<b>CY + 1</b>						
Control	4.5 ± 4.5	13.0 ± 2.0	9.0 ± 4.7	16.2 ± 5.1	23.1 ± 3.2	10.6 ± 3.0
CY control	1.8 ± 2.8	14.8 ± 1.7	11.5 ± 2.2	4.0 ± 5.0	15.9 ± 1.9	9.4 ± 4.2
Ds BGG	0.8 ± 1.9	8.5 ± 4.2	3.7 ± 4.1	8.3 ± 9.3	1.8 ± 4.5	0
CY-Ds BGG	2.2 ± 3.7	11.9 ± 1.7	10.4 ± 2.7	6.4 ± 3.8	13.9 ± 2.1	6.9 ± 4.7
Ds PPD	1.3 ± 3.1	2.8 ± 3.1	0	15.7 ± 3.6	15.8 ± 2.8	0
CY-Ds PPD	2.5 ± 3.3	8.8 ± 1.8	5.5 ± 4.8	4.1 ± 5.2	16.3 ± 2.1	8.9 ± 6.2
<b>CY - 3</b>						
Control	0	13.8 ± 2.1	6.2 ± 4.4	10.4 ± 10.4	22.8 ± 6.0	7.1 ± 7.1
CY control	1.7 ± 3.9	15.4 ± 2.0	10.3 ± 3.6	4.9 ± 6.2	19.3 ± 2.1	6.2 ± 6.8
Ds BGG	0	4.6 ± 5.6	3.6 ± 4.0	6.3 ± 8.4	4.6 ± 5.6	0
CY-Ds BGG	3.0 ± 4.7	11.6 ± 3.5	8.1 ± 4.4	1.8 ± 4.6	6.4 ± 6.4	0
Ds PPD	0	1.0 ± 2.1	0	15.6 ± 2.8	11.3 ± 8.6	3.8 ± 6.8
CY-Ds PPD	0	3.3 ± 2.7	0	6.3 ± 7.0	19.3 ± 2.2	11.5 ± 6.6

\* Reaction size expressed as mean diameter of a minimum of six animals ± SD.



**Figure 3.** Effect of CY + 1 on specific desensitization. Skin test reactivity as measured by size of reaction to (a) OA, (b) BGG and (c) PPD, 8 days after immunization. Animals in (a) were immunized with OA in FCA and those in (b) and (c) with BGG in FCA. The notation ○—○ refers to control animals when skin tested in (a) with OA, in (b) with BGG and in (c) with PPD. One group received two s.c. injections of OA (x—x), another two s.c. injections of BGG (□—□) and the third two s.c. injections of PPD (●—●). Other groups of animals were set up in parallel having been given 300 mg/kg CY 1 day after immunization (○---○, x---x, □---□ and ●---●, respectively).

given CY 1 day after immunization (Fig. 3,  $P < 0.001$  for all three antigens). In contrast, specific desensitization was unaltered in guinea-pigs given CY 3 days before immunization (Tables 1 and 2). As reported previously (Uhr & Pappenheimer, 1958; Dwyer & Kantor, 1973), specific desensitization strongly inhibits the delayed hypersensitivity reaction. In addition, we noted that the 4 hr Arthus reactions were markedly suppressed in animals desensitized with OA or BGG.

## DISCUSSION

Desensitization in these experiments has been found to occur following the injection of a large amount of antigen. This inhibited the delayed hypersensitivity reaction to that antigen and to another antigen to which the animal had been immunized. The mechanisms responsible for both the specific and non-specific anergy are unknown. Previous work has suggested that 'non-specific desensitization' differs from specific in that a radiosensitive immunoregulatory mechanism, that is probably T-cell dependent, is involved (Thestrup-Pedersen *et al.*, 1977). On the other hand specific desensitization, which is not radiosensitive, has been attributed to simpler mechanisms such as macrophage inactivation (Poulter & Turk, 1976) production of specific antibody (Liew, 1975) and the stimulation of suppressor cells to liberate blocking factors (Dwyer & Kantor, 1975).

CY provides a valuable tool for exploring all forms of regulatory mechanisms in delayed hypersensitivity. Given 3 days before immunization CY produces enhancement of many delayed hypersensitivity reactions. In addition, CY given 1 day after immunization, can prevent the suppression of delayed hypersensitivity reactions by antigenic competition, (Dwyer *et al.*, 1981). Also, 'tolerance' to the contact sensitizer dinitrochlorobenzene can be broken by CY (Polak & Turk, 1974; Polak, Geleick & Turk, 1975; Sommer, Parker & Turk, 1975). These data strongly suggest that the breaking of 'tolerance' and the enhancement of the delayed hypersensitivity reactions, in these CY-treated animals, is due to the elimination of active suppressor cells or their precursors (Katz, Parker, Sommer & Turk, 1974). If desensitization requires intact suppressor-cell mechanisms then it should not be possible to induce desensitization in guinea-pigs treated with CY.

Our data demonstrates that this is the case in animals immunized with OA or BGG in FCA when 'non-specifically desensitized'. The production of 'non-specific desensitization' by both OA or BGG was prevented when CY was given either before or after immunization. Although little work has been done using PPD as a desensitizing antigen, in our experiments it produced good 'non-specific desensitization' of delayed hypersensitivity to OA and BGG. This was also susceptible to CY treatment. The demonstration that 'non-specific desensitization' to an array of antigens of different strengths can be prevented by CY

treatment suggests that this may be a general phenomenon.

Our data confirms the concept that 'non-specific desensitization' differs from specific, as specific desensitization is not inhibited in animals given CY 3 days before immunization. However, given 1 day after immunization, CY prevents specific desensitization. These results suggest that a suppressor cell, activated by immunization and susceptible to CY, might at least be partially responsible for specific desensitization. This suppressor cell is presumably different from that involved in the enhancement of delayed hypersensitivity, as the precursors of these latter cells are highly sensitive to CY. However, the precursors of the suppressor cells involved in specific desensitization are not susceptible to CY as specific desensitization is unaltered by CY given 3 days before immunization. As specific desensitization induces stronger depression of the delayed hypersensitivity reaction than that induced by 'non-specific desensitization', it could be postulated that if suppressor cells are involved they may be antigen specific. Our data would support this as CY only breaks desensitization when given after immunization. However, an alternative is that the suppressor cells, which are geographically close to the cells they inhibit, produce a non-specific factor. Such geographical proximity might explain the greater difficulty in destroying their action with CY and hence the need to give CY at a later time.

This paper, therefore, adds a fourth system in which CY-sensitive suppressor cells or their precursors would seem to be involved in the regulation of the immune response. The first is regulation of the normal immune response, the second is 'tolerance' to chemical sensitizers and the third is antigenic competition. The CY-sensitive cells involved in antigenic competition and non-specific desensitization would seem to differ from those involved in 'tolerance', specific desensitization and the normal immune response in being antigenically non-specific.

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