

## DEPRESSION OF GRASS POLLEN-INDUCED LYMPHOCYTE TRANSFORMATION BY SERUM FROM HYPOSENSITIZED PATIENTS

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### SUMMARY

Serum from grass-sensitive patients who had received hyposensitization therapy was found able to depress the *in vitro* response to *Holcus lanatus* grass pollen extract of lymphocytes from untreated grass-sensitive individuals.

The inhibitory activity could no longer be demonstrated at serum dilutions equal to or greater than 1/100 and was not seen when the cells were stimulated with PHA or unrelated antigens. It was contained in the precipitated fraction of 33% saturated ammonium sulphate-treated serum, segregated with the 7S peak of Sephadex G-200 and with the IgG fraction of DEAE-Sephadex-fractionated serum and could be removed by the absorption of the serum with the *Holcus lanatus* allergenic extract.

These data suggest that the serum factor responsible for the depressed *in vitro* lymphocyte response to the allergen in hyposensitized patients was a 7S IgG antibody induced by immunotherapy.

### INTRODUCTION

In a previous paper we showed that lymphocytes from grass-sensitive individuals transform *in vitro* in the presence of grass pollen allergenic extracts, whereas lymphocytes from non-atopic subjects do not respond *in vitro* to the same extracts. Moreover lymphocytes from hyposensitized patients showed a less marked response in comparison to that of cells from untreated grass-sensitive patients (Romagnani *et al.*, 1973).

The experiments presented in this paper were performed in order to understand the significance of this phenomenon and to ascertain the nature of the factor(s) responsible for the depressed lymphocyte response in atopic patients who had received hyposensitization therapy.

## MATERIALS AND METHODS

*Lymphocyte cultures*

Forty millilitres of venous heparinized blood were divided into 4-ml aliquots and carefully layered on top of equal volumes of fresh filter-sterilized Ficoll-Urovison gradient (specific gravity 1.077). The gradient and cell suspension was centrifuged for 30 min at 400 *g* (at interface) at ambient temperature. The band of cells at the plasma-gradient interface was carefully aspirated with a pipette and then washed twice with NCTC 109 medium. This routinely gave 80–95% lymphocytes. The cells were then counted and adjusted at  $0.5 \times 10^6$ /ml in medium containing 15% of selected plasma or serum. The suspension was distributed in 2-ml aliquots in plastic tubes (100 × 16 mm) and the stimulants added in 0.1-ml volumes. A part of the experiments was performed by the 'microplate' technique (Janossy & Greaves, 1971) using V-bottom plates (Greiner, Nürtingen). When this method was used  $0.2 \times 10^6$  lymphocytes in 0.2 ml of 10% serum and appropriate stimulant containing NCTC 109 medium were distributed in each well. The plates were then incubated in a 5% CO<sub>2</sub> humidified atmosphere at 37°C.

Sixteen hours before harvesting the tube and well cultures were pulsed with 0.1 and 0.05  $\mu$ Ci of [<sup>14</sup>C]thymidine (Radiochemical Centre, Amersham), respectively. All the cultures were always performed in triplicate. The contents of both plastic tubes and microplate wells was collected on glass fibre paper filters (2.5 cm GF/B, Whatman) by a 'multiple automated sample harvester'. Essentially, the function of this machine was to remove the content of the tubes or wells and wash the filters, thus eliminating the unbound thymidine from the filters. The washings were performed with saline, 5% TCA and methanol. Following the harvesting procedure the filters were dried in a drying oven at 60°C for 2 hr and then placed in polyethylene scintillation vials containing 10 ml of Instagel (Packard). After cold and dark adaptation of the vials, the thymidine incorporation was determined by a Packard 2425 liquid scintillation spectrophotometer.

*Lymphocyte stimulants*

PHA M (Difco) was diluted with 5 ml of NCTC 109 medium and added at a concentration of 0.05 ml/ml.

PPD (Sclavo, Siena) was employed at concentrations ranging between 0.5 and 5  $\mu$ g/ml.

Semipurified streptococcal M protein (Sclavo, Siena) obtained from type 1 beta-haemolytic streptococci by the method of Lancefield & Perlman (1952) was used at a concentration of 5  $\mu$ g/ml.

*Holcus lanatus* (HL) grass pollens were weighed, defatted, and extracted for 24 hr at 4°C with ten times their weight of 0.125 M NH<sub>4</sub>HCO<sub>3</sub>. The extract was dialysed in Visking 18/32" diameter dialysis tubing against several changes of 0.002 M NH<sub>4</sub>HCO<sub>3</sub>, and finally against distilled water and freeze-dried. Concentrations of the extract ranging between 0.015 and 150  $\mu$ g/ml (Lowry method) were used.

*Radioallergosorbent test (RAST)*

Specific IgE against HL allergenic extract were measured by a modified RAST method (Sarsfield & Gowland, 1973) which was found to give sufficiently accurate quantitation of serum reaginic antibodies (Ricci, Biliotti & Romagnani, 1974).

*Haemagglutination procedure*

Anti-HL haemagglutinating antibodies were detected by testing the sera against SRBC treated with tannic acid (1/20,000) and sensitized with the dialysed HL extract (300 µg/ml of 2.5% SRBC).

*Immuno-electrophoresis*

The micro-method described by Scheidegger (1955) was used.

*Human sera*

Normal pooled serum (NS) was obtained from twenty normal male volunteers chosen from medical students.

Atopic serum (AS) was obtained from twelve grass-sensitive patients suffering from rhinitis and/or bronchial asthma supported by a seasonal history of symptoms in May and June.

Serum from hyposensitized patients (HS) was obtained from twelve grass-sensitive patients, who had received immunotherapy for 3–4 months (20,000–40,000 PNU), 15 days after the last aqueous extract treatment injection. The sera were pooled, filter-sterilized, divided in convenient aliquots and stored at  $-20^{\circ}\text{C}$  until use.

*Fractionation of sera*

Five-millilitre aliquots of H serum were precipitated with 40% saturated  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was dissolved with saline and precipitated again with 33% saturated  $(\text{NH}_4)_2\text{SO}_4$ . This precipitate and the first supernatant were dialysed against several changes of PBS and then against NCTC 109 medium, concentrated to the original volume of the unfractionated serum by vacuum dialysis, and filter-sterilized.

In another experiment the A and H serum were fractionated on a Sephadex G-200 column by using PBS as elution medium. The fractions corresponding to the mid-portion of 19S, 7S and 4S peaks were collected. The H serum was also fractionated on DEAE-Sephadex A-50 by the method described by Perper *et al.* (1967), using 0.01 M phosphate buffer (pH 7.8) to isolate the IgG fraction. The molarity of the buffer was then increased to 0.5 M NaCl in order to recover the remaining serum fractions. The content of these fractions was confirmed by immuno-electrophoresis. Each fraction was dialysed against PBS and then NCTC 109 medium, reconstituted to the original volume and filter-sterilized.

*Immunosorbent technique*

Microcrystalline cellulose was activated with CNBr according to the method described by Wide (1969) and sensitized with HL extract and HSA at a concentration of 10 mg (dry weight)/100 mg of cellulose. Two different aliquots of the H serum (10 ml) were then incubated with vertical rotation for 24 hr with 200 mg of HL- and HSA-sensitized cellulose. The tubes were then centrifuged and serum samples incubated again with vertical rotation for 12 hr with 200 mg of fresh HL- and HSA-sensitized cellulose. After centrifuging the sera were dialysed against PBS and NCTC 109 medium, filter-sterilized and stored in little aliquots at  $-80^{\circ}\text{C}$  until use.

**RESULTS***(1) Lymphocyte response to HL extract in untreated and treated grass-sensitive patients*

When lymphocytes from untreated and treated grass-sensitive patients were cultured in

autologous plasma in the presence of a single concentration (15  $\mu\text{g/ml}$ ) of HL extract, the response of cells from hyposensitized patients appeared to be less marked than that of untreated atopic subjects (Table 1).

TABLE 1. *In vitro* lymphocyte response (measured by [ $^{14}\text{C}$ ]thymidine incorporation) to *Holcus lanatus* (HL) grass pollen extract in untreated and treated grass-sensitive patients\*

	Number of patients	Ct/min		Stimulation index
		No HL	HL	
Untreated	13	124 $\pm$ 72	958 $\pm$ 701	9.0 $\pm$ 6.7
Treated	7	158 $\pm$ 125	352 $\pm$ 341	3.4 $\pm$ 3.4
<i>P</i> value		n.s.	<0.01	<0.01

\*The cells were incubated for 6 days in plastic tubes in the presence of 15% autologous plasma. HL extract (15  $\mu\text{g/ml}$ ) was added to stimulate the cells.

### (2) Effect of sera from hyposensitized patients on lymphocyte response to HL extract, other unrelated antigens and PHA

When lymphocytes from nine untreated grass-sensitive patients were washed three times and stimulated *in vitro* by HL extract in the presence of 'atopic' (A) and 'hyposensitized' (H) serum a less marked response in the presence of H serum was constantly observed. On the contrary, the lymphocyte response to PHA and two unrelated antigens (PPD and streptococcal M protein) was not significantly different in the presence of A or H serum (Fig. 1).

### (3) Reaginic and precipitating antibodies in A and H serum

Reaginic and precipitating antibodies in A and H serum were investigated by RAST

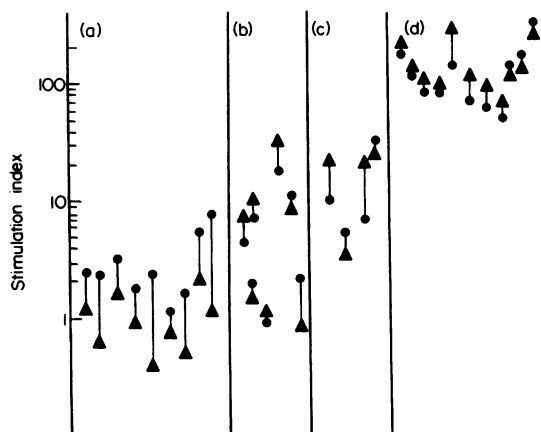


FIG. 1. *In vitro* response (measured by [ $^{14}\text{C}$ ]thymidine incorporation) to (a) *Holcus lanatus* grass pollen extract, unrelated antigens PPD(b) and streptococcal M protein (c) and PHA (d) of lymphocytes from grass-sensitive patients cultured in the presence of A (●) and H (▲) serum. A statistically significant ( $t = 2.62$ ;  $P < 0.01$ ) difference between the response of the cells in the presence of A and H serum was observed only when HL extract was used as stimulant.

and haemagglutination procedure, respectively. Thus, it was found that H serum contained a greater amount of HL-specific IgE than A serum and showed a higher titre of haemagglutinating antibodies (Table 2). By fractionation of the H serum on Sephadex G-200 and

TABLE 2. IgE reaginic and IgG haemagglutinating antibodies in A and H serum

Serum	RAST values* (‰)	Haemagglutinating antibody titre
A	100	< 1:10
H	400	1:320

\*Parts per thousand of reference reaginic serum.

DEAE-Sephadex column, the anti-HL haemagglutinating activity was found to be related to the 7S IgG immunoglobulins.

#### (4) Titre of inhibitory activity in H serum

The H serum was diluted in normal pooled serum and ten-fold serial dilutions were tested on lymphocytes from grass-sensitive patients stimulated with HL extract. The inhibitory activity of H serum was no longer demonstrated when the serum was 100 times diluted in respect to the normal pooled serum (Fig. 2).

#### (5) Effect of precipitation of H serum with ammonium sulphate

When  $(\text{NH}_4)_2\text{SO}_4$  precipitated and supernatant fractions of H serum were tested on lymphocyte cultures, the inhibitory activity was constantly found to be present in

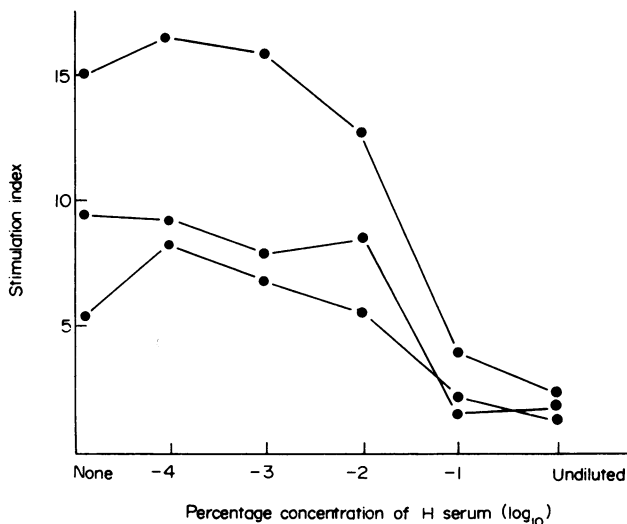


FIG. 2. Titre of inhibitory activity in H serum. Lymphocytes from three grass-sensitive individuals were stimulated by HL extract in the presence of decreasing amounts of H serum diluted in normal pooled serum.

the precipitated fraction. The first supernatant fraction was no longer inhibitory (Table 3).

TABLE 3. Effect of H serum on lymphocyte response (measured by [ $^{14}$ C]thymidine incorporation) to HL extract after saturated ammonium sulphate precipitation

Test fraction*	Stimulation index		
	Experiment 1	Experiment 2	Experiment 3
Normal serum (15%)	9.7	8.0	2.8
Normal serum (10%)	8.4	6.3	2.9
HS	4.1	3.0	1.3
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitate	5.9	3.8	1.6
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> surnatant	7.2	9.0	2.1

\* H serum (HS) was precipitated, and supernatant fractions of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-treated H serum were added in the proportion of 5%. Ten per cent normal serum was always present.

(6) *Effect of fractionation of H serum on Sephadex G-200 and DEAE-Sephadex*

The inhibitory activity of Sephadex G-200 fractionated H serum was demonstrable in the 7S peak fractions, whereas none of the fractions obtained by gel-filtration on Sephadex G-200 of A serum appeared to be inhibitory. The inhibitory activity of H serum was also present in the IgG fraction of DEAE-Sephadex and was absent from the H serum fraction from which most of the IgG had been removed (Table 4).

(7) *Absorption of H serum inhibitory activity by specific antigen*

When H serum was incubated with HL-sensitized CNBr-activated microcrystalline cellulose it was found that the anti-HL haemagglutinating antibody titre was reduced from 1/320

TABLE 4. Effect of H serum fractions on lymphocyte response (measured by [ $^{14}$ C]thymidine incorporation) to HL extract after filtration on Sephadex G-200 and DEAE-Sephadex

Test fraction*	Stimulation index				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
NS alone	2.8	2.8	4.0	6.4	2.7
AS	3.4	2.4	4.1	8.0	2.5
HS	1.2	1.0	1.2	3.7	1.7
AS					
19S	—	3.6	2.9	6.2	2.7
7S	3.1	2.5	3.5	5.5	2.2
4S	3.0	2.5	3.4	6.0	2.3
HS					
19S	—	2.9	4.8	6.1	2.5
7S	1.5	1.6	2.2	3.6	1.7
4S	2.8	2.9	2.8	6.2	2.5
IgG fraction	—	1.3	2.1	—	—
IgG-depleted fraction	—	2.0	4.1	—	—

\* All the cultures contained 10% normal pooled serum (NS) and 5% of test fraction.

to  $<1/10$ , whereas the incubation of the same serum with HSA-sensitized cellulose did not induce any modification of the antibody titre. By testing the HL- and HSA-absorbed sera on lymphocyte cultures stimulated by HL extract it was possible to demonstrate that in the presence of antibody-deprived H serum a more marked uptake of labelled thymidine occurred, or, as in experiment 4, a 10-fold lower concentration of antigen was sufficient to activate the cells (Fig. 3).

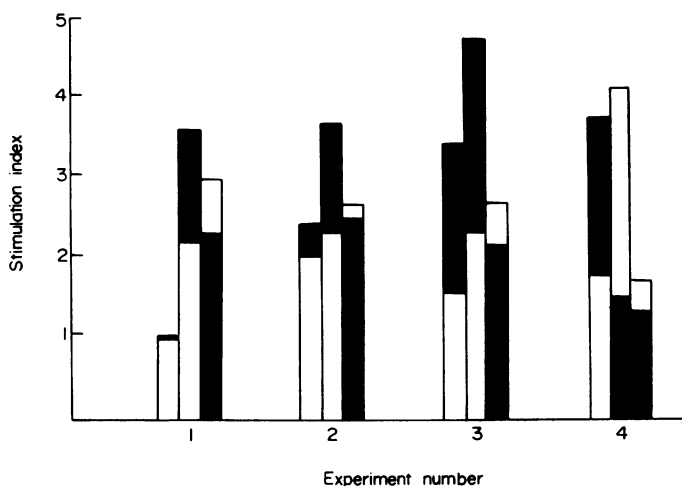


FIG. 3. Effect on lymphocyte response to HL extract of anti-HL antibody rich (blank areas) and deprived (solid areas) H serum. Each column represents the response of cells to a single concentration of HL extract (1.5, 15 and 150  $\mu\text{g/ml}$  from left to right in each experiment).

## DISCUSSION

*In vitro* lymphocyte response to allergenic extracts has been found to be a useful test for the study of immune responses in atopic patients (Malley *et al.*, 1971; Rocklin *et al.*, 1973).

In unpublished experiments we showed that the lymphocyte stimulating capacity of HL extract was related to the allergenic rather than non-allergenic constituent(s) of the same extract. Moreover, by testing *Dermatophagoides pteronyssinus* fractions obtained by isofocusing preparative electrophoresis we were able to demonstrate the presence of two different RAST-inhibiting fractions which also appeared to be able to activate lymphocytes from mite-sensitive patients (Ricci, 1974). Thus, *in vitro* lymphocyte response to allergenic extracts seems to reflect the recognition of allergenic components of the same extracts by sensitized lymphocytes.

The experiments presented in this paper show that sera from patients who were allergic to grass pollens and who had received hyposensitization therapy were able to depress grass pollen-induced lymphocyte transformation. This inhibitory activity was not observed when the cells were stimulated with PHA or unrelated antigens and could no longer be demonstrated at serum dilutions equal or greater than 1/100. This activity was contained in the precipitated fraction of 33% saturated ammonium sulphate-treated serum, in the 7S peak of Sephadex G-200 and in the IgG fraction of DEAE-Sephadex-fractionated serum and could be removed by the absorption of the serum with the allergenic extract used to

stimulate the cells. Thus, it is likely that the serum factor responsible for the depressed lymphocyte response was a 7S IgG antibody induced by immunotherapy.

The modulation of *in vitro* lymphocyte transformation by antibodies has been extensively investigated by Oppenheim (1972) in animals. These findings indicated that an excess of soluble antibodies can block the response of sensitized lymphocytes to specific antigen, due either to a peripheral mechanism of preventing the antigen from stimulating the lymphocytes (Jacobs, Blaese & Oppenheim, 1972), or based on a central inactivation of the lymphocytes (Feldman & Diener, 1970). Our data in man seem to be in agreement with those obtained in animals. However, the relationship of the *in vitro* observations to the *in vivo* effects of antibody on lymphocyte is unclear, both in animal and man.

Although the induction of cell-mediated immune responses can be blocked readily by simultaneous or prior treatment with antibody (Oppenheim & Seeger, 1972) it is very difficult to inhibit established thymic-dependent *in vivo* reactions by the passive administration of antibodies (Uhr & Moller, 1968). On the other hand, it has been shown that the PFC response by spleen lymphocytes was inhibited by the passively administered antibody which did not act directly on the lymphoid cell population but neutralized the antigenic stimulus by combining with the antigen product of macrophages within or on the surface of the macrophage (Pierce, 1969).

The homocytotropic antibody response can be suppressed in the rat by the passive administration of IgG antibody (Tada & Okumura, 1971). However, in mice, pre-existing levels of reaginic antibodies are not reduced, and secondary response of antibodies of this class is not suppressed by passive administration of homologous IgG (Ishizaka & Okudaira, 1972). More recently an inhibitory cell interaction in the homocytotropic antibody formation has been demonstrated in the rat. This inhibition was mediated by a subcellular component of primed T cells which was not immunoglobulin in nature, but showed an affinity and specificity to the carrier molecule (Tada, Okumura & Taniguchi, 1973).

In man it has been clearly demonstrated that serum-blocking IgG antibody, measured by inhibition of antigen-induced leucocyte histamine release is increased 20- to 40-fold after preseasonal immunotherapy (Levy *et al.*, 1971). There is a suggestive but no definite relationship between the degree of improvement and the IgG antibody level in these patients. Moreover, other immunological and cellular changes, such as lowered reaginic antibody titres and decreased leucocyte sensitivity have been reported to accompany the therapy of pollen allergy (Levy *et al.*, 1971; Melam *et al.*, 1971; Johansson *et al.*, 1974).

These data suggest that the clinical improvement following specific immunotherapy must be the result of complex changes in the immunological and cellular components of allergic disease. Thus, it could be suggested that the production of IgG antibody and perhaps other non-immunoglobulin soluble factors may play an important role on the regulation and control of IgE antibody response and then on clinical improvement following long-term immunotherapy.

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