

The site of action of antigen D immunotherapy

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Attempts to determine the mechanism and site of action of antigen D immunotherapy involve the culture of gelatin-separated human peripheral lymphocytes with varying concentrations of timothy allergens. Lymphocytes obtained from patients at different stages of treatment were cultured for 6 days, and the degree of reactivity was quantitated by ^3H -thymidine uptake of the antigen-reactive cells. These studies suggest that in vitro lymphocyte transformation may serve as a good index for the effectiveness of antigen D immunotherapy. The site of action and the mechanism of antigen D immunotherapy are discussed.

Over the last three years, approximately 200 timothy-sensitive patients have been treated with either the dialyzed aqueous extract (WST) or a low molecular weight fraction (antigen D) of timothy pollen in an alginate suspension. These studies^{1, 2} demonstrated that patients receiving antigen D immunotherapy required less medication and reported significantly fewer clinical symptoms than patients treated with WST. A comparison of the reaginic antibody titers at various stages during immunotherapy with either WST or antigen D revealed that patients treated with WST in alginate showed no significant change in the reagin titers. On the other hand, the majority of patients treated with antigen D showed significant decreases in the reaginic antibody titers.² Fig. 1 illustrates the pattern of changes in reagin titers measured by Prausnitz-Küstner tests in both groups of treated patients. During the early phase of treatment, antigen D-treated patients generally showed a drop in reagin titers which stabilized at some low level and remained there throughout the treatment. The initial drop in reagin titer may reflect in vivo neutralization of circulating reagin. However, the maintenance of these reduced reagin titers over such a prolonged period suggests a suppression or turning off of reaginic antibody production.

Studies in our laboratory with rhesus peripheral lymphocytes demonstrated that antigen-induced lymphocyte transformation is a good index of the state of hypersensitivity in the rhesus monkey.³⁻⁵ Therefore, we explored the lymphocyte transformation of peripheral lymphocytes obtained from timothy-sensitive patients to: (1) determine whether this method provides a less subjective index of the effectiveness of immunotherapy than daily diaries, and (2) explore the mechanism or site of action of antigen D immunotherapy.

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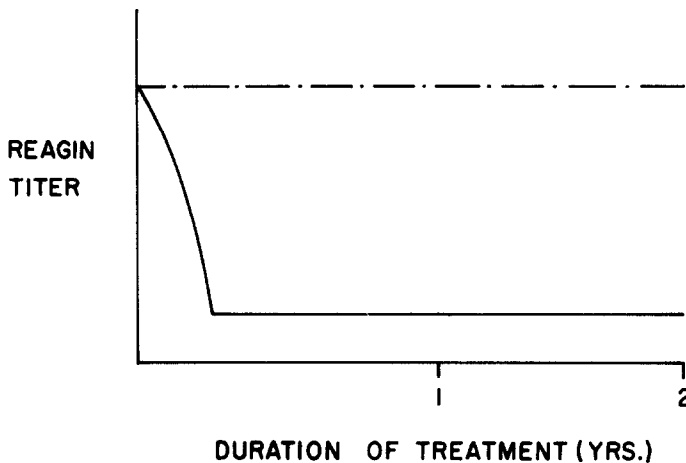


FIG. 1. Pattern of reagin titer in patients treated with timothy pollen fractions. WST treated — · — · — · — · — · —; antigen D treated —————.

The present study suggests that: (1) antigen-induced lymphocyte transformation provides a suitable index of the effectiveness of antigen D immunotherapy, and (2) the apparent suppression of reaginic antibody production is due to the blockage of receptor sites on antigen-reactive cells by antigen D.

MATERIALS AND METHODS

Antigens and mitogens

The dialyzed aqueous extract of timothy grass pollen (WST) and a low molecular fraction of timothy pollen extract (antigen D) were prepared as previously reported.⁶ Protein concentrations were determined by the method described by Campbell and associates.⁷ A purified preparation of phytohemagglutinin (PHA) was provided by Dr. D. Rigas (University of Oregon Medical School) and was used as a nonspecific mitogen in all lymphocyte transformation experiments.

Lymphocyte purification

Approximately 30 ml. of blood was collected in plastic syringes containing phenol-free heparin (100 units per 50 ml. of blood). After an hour incubation at 37° C., an equal volume of sterile 3 per cent gelatin in saline was mixed with the blood in a 100 ml. Nalgene tube. This mixture was incubated at 37° C. for 60 to 90 minutes, and the leukocyte-rich supernatant was removed, diluted 1:4 with warm Eagle's minimum essential medium (MEM), and centrifuged at 250 g for 15 minutes at 37° C. The pelleted cells were washed once in warm MEM, resuspended in MEM, and counted in a hemacytometer.

Lymphocyte cultures

Lymphocytes were diluted to a concentration of 2×10^6 mononuclear cells per milliliter in MEM enriched with 20 per cent homologous serum (from nonallergic donors), glutamine (29 mg. per milliliter), nonessential amino acids, and MEM vitamin solution, 100 X (0.1 ml. per 100 ml. of culture fluid), penicillin (2,500 units per milliliter), and streptomycin (2,500 units per milliliter). Two-milliliter portions of this stock suspension were dispensed into Wallis-Melnick screw-cap culture tubes (15 mm. $\times$ 125 mm.). The antigens (0.1 μ g to 2.5 mg. protein WST or antigen D) or PHA (10 μ g protein) were added in a 0.1 ml. volume, and the tubes were sealed and incubated on a roller apparatus (< 1 r.p.m.) at 37° C. for 6 days. Twenty-four hours before harvesting, 0.1 ml. of tritiated thymidine ($^3\text{H-Td}$,

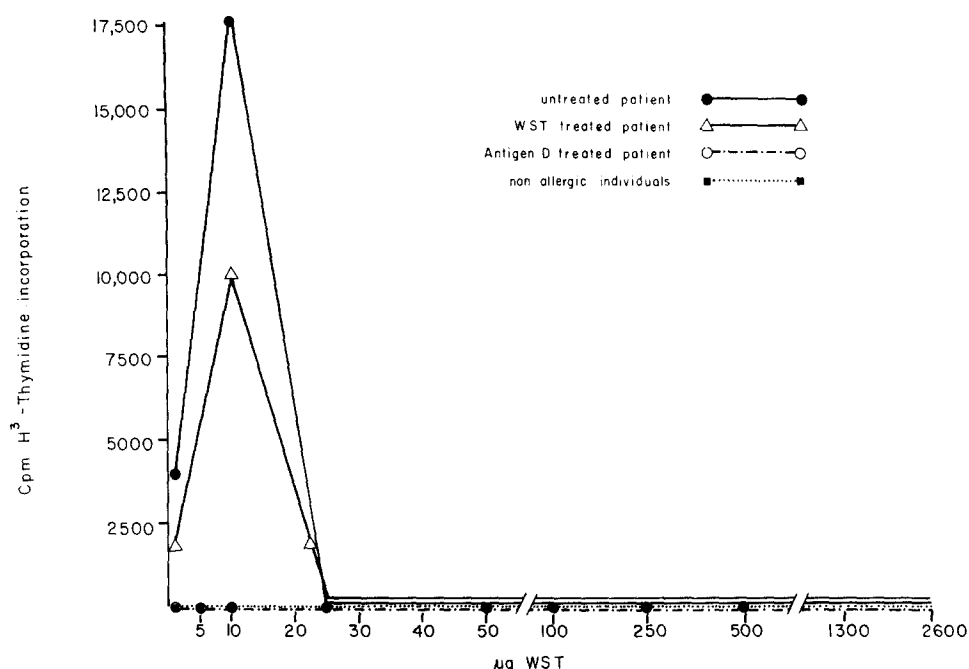


FIG. 2. Lymphocyte transformation responses of cells obtained from timothy-sensitive patients receiving no treatment or WST, or antigen D immunotherapy. Lymphocytes obtained from nonallergic individuals cultured with either WST or antigen D served as negative controls. Each point represents the mean incorporation of ^3H -thymidine of triplicate tubes and was corrected for ^3H -thymidine incorporation of cells cultured without WST (experimental control). Individual values varied ± 15 per cent from the mean.

New England Nuclear Corp., specific activity 5.7 c per mM.) was added to a final concentration of $1 \mu\text{c}$ per milliliter. When harvested, the cells were pelleted at 250 g for 15 minutes at 3°C . and washed three times in ice-cold 0.9 per cent saline. The level of thymidine incorporation was determined by the liquid scintillation methods (Packard liquid scintillation counter) as previously described.^{4, 6} Each experiment was done in triplicate, and the agreement between levels of stimulation in triplicate cultures varied 5 to 25 per cent from the mean. In most cases, the individual values of triplicate cultures varied less than 15 per cent from the mean.

Selection of patients

Heparinized blood samples were obtained from randomly selected timothy-sensitive patients receiving: (1) antigen D immunotherapy, (2) WST immunotherapy, or (3) no treatment during the last pollen season (1970), and (4) from nonallergic volunteers. Most of the allergic patients studied in these experiments experienced clinical symptoms from many other allergenic materials, such as epidermals, house dust, foods, insects, tree pollen, and other grasses. All blood samples obtained for this study were collected one to 3 months after the last treatment injection or inhalant exposure of the patient to timothy pollen (July 15 to October 15). Clinical evaluation was based on daily diaries.

RESULTS

Fig. 2 shows the general pattern of lymphocyte transformation responses of cells obtained from timothy-sensitive patients and nonallergic volunteers

TABLE I. Relationship between reagin titer and in vitro lymphocyte transformation response of timothy-sensitive patients

Patient	³ H-thymidine incorporation* (c.p.m.)	Passive transfer titer†
R. B.	18,000	500
G. A.	2,700	10
L. B.	8,400	50
L. H.	5,300	500
S. W.	7,300	100
L. T.	2,950	50
C. W.	6,900	100
L. L.	8,850	100
R. G.	3,900	10
K. B.	3,750	10

*Corrected for incorporation of ³H-thymidine into cells cultured without allergen (WST).

†Sera dilutions implanted into the backs of nonallergic volunteers challenged 48 hours later with 10⁻⁶ Gm. WST. Tests done in duplicate and indicated titers represent highest dilution of sera giving a 5 to 10 mm. diameter wheal reaction.

TABLE II. Lymphocyte transformation response of cells obtained from nonallergic individuals and timothy-sensitive patients incubated with allergen in vitro

Number of patients	Previous treatment	Range of ³ H-thymidine incorporation* (c.p.m.)
15	Untreated	2,700 - 18,500
15	WST	2,500 - 7,800
10	Nonallergic	No response
20	Antigen D	No response

*Corrected for incorporation of ³H-thymidine into cells cultured without allergen (WST). Results represent the mean incorporation observed in triplicate tubes. Individual values varied $\pm$ 15 per cent from mean values.

one to 3 months after the last treatment injection or inhalant exposure to timothy pollen. Ninety to ninety-five per cent of the patients tested showed maximum stimulation of their peripheral lymphocytes with 10 μ g protein of WST. The few patients who failed to show maximum stimulation at 10 μ g WST usually showed peak responses at either 100 or 250 μ g protein WST. Lymphocytes obtained from a number of patients showed maximum stimulation peaks at more than one antigen concentration (10 μ g and either 100 or 250 μ g WST). In all cases, however, the maximum stimulation peak at 10 μ g WST was several times higher than the responses at higher antigen concentration.

Lymphocytes obtained from WST-treated and untreated timothy-sensitive patients showed various degrees of transformation, with tritiated thymidine incorporation levels reaching as high as 18,000 c.p.m. The level of transformation, however, did not correlate with the P-K titer of an individual patient (Table I) but may be related to his level of clinical symptoms. Lymphocytes obtained from patients treated with either antigen D (one or more years) or from nonallergic individuals showed no transformation at any of the allergen concentrations tested (0.1 μ g to 2.5 mg. protein WST). They did, however, respond normally to a nonspecific mitogen such a PHA.

Lymphocytes obtained from patients reported in this study did not respond in vitro to antigen D at any concentration tested (0.1 μ g to 2.5 mg. protein).

A summary of our lymphocyte transformation results is shown in Table II. All tests of the untreated timothy-sensitive patients showed some level of stimulation, the majority between 2,700 and 10,000 c.p.m. Patients receiving WST immunotherapy (aqueous or alginate suspension) showed transformation levels mainly within the range of 2,500 to 7,800 c.p.m. In contrast, none of the 10 normal subjects (nonallergic) or 20 patients treated with antigen D for one or more years showed any incorporation of tritiated thymidine upon exposure to either antigen D or WST (0.1 μ g to 2.5 mg. protein).

DISCUSSION

Attempts to demonstrate a relationship between the P-K titer and the level of in vitro lymphocyte transformation of individual patients were unsuccessful (Table I). Clinical evaluation of the patients studied during the completed pollen season indicated that antigen D-treated patients had fewer symptoms and required less medication than either WST-treated or untreated timothy-sensitive patients. The observation that lymphocytes obtained from patients receiving antigen D immunotherapy do not respond to antigen (WST or antigen D) in culture suggests a possible correlation between the degree of clinical symptoms and a patient's in vitro lymphocyte transformation response. Experiments are currently in progress to follow the lymphocyte transformation response of individual patients at various stages during immunotherapy with either WST or antigen D to determine if this approach will provide an index for the effectiveness of immunotherapy.

Previous studies by Richter and Naspitz⁸ and others⁹⁻¹¹ demonstrated that patients with atopic hypersensitivity to a variety of allergens show antigen-induced lymphocyte transformation. Generally the level of antigen required to achieve maximum in vitro stimulation was significantly higher (100 to 500 μ g protein) in these reports than it was in our own studies. The low level of antigen (10 μ g protein) required to obtain maximum stimulation suggests that either the patients possessed a larger number of cells capable of recognition and response to antigen or the binding affinity of the recognition units on the responding cells was very high. The observation that a number of patients showed peak responses at more than one antigen concentration (10 μ g and either 100 or 250 μ g protein) suggests that the concentration of antigen required for maximum stimulation reflects the binding affinity of the antigen to the recognition units of the responding cells. Consequently, those timothy-sensitive patients whose lymphocytes show in vitro transformation only at high concentrations of antigen (100 to 250 μ g protein) may represent individuals whose cells have recognition units with low binding affinity for antigen and who probably would not show clinical improvement with either WST or antigen D immunotherapy.

The mechanism of in vitro transformation of lymphocytes has been clarified by recent studies using antilymphocyte sera (ALS), and its bivalent and monovalent fragments.¹²⁻¹⁵ These studies revealed that the intact antibody

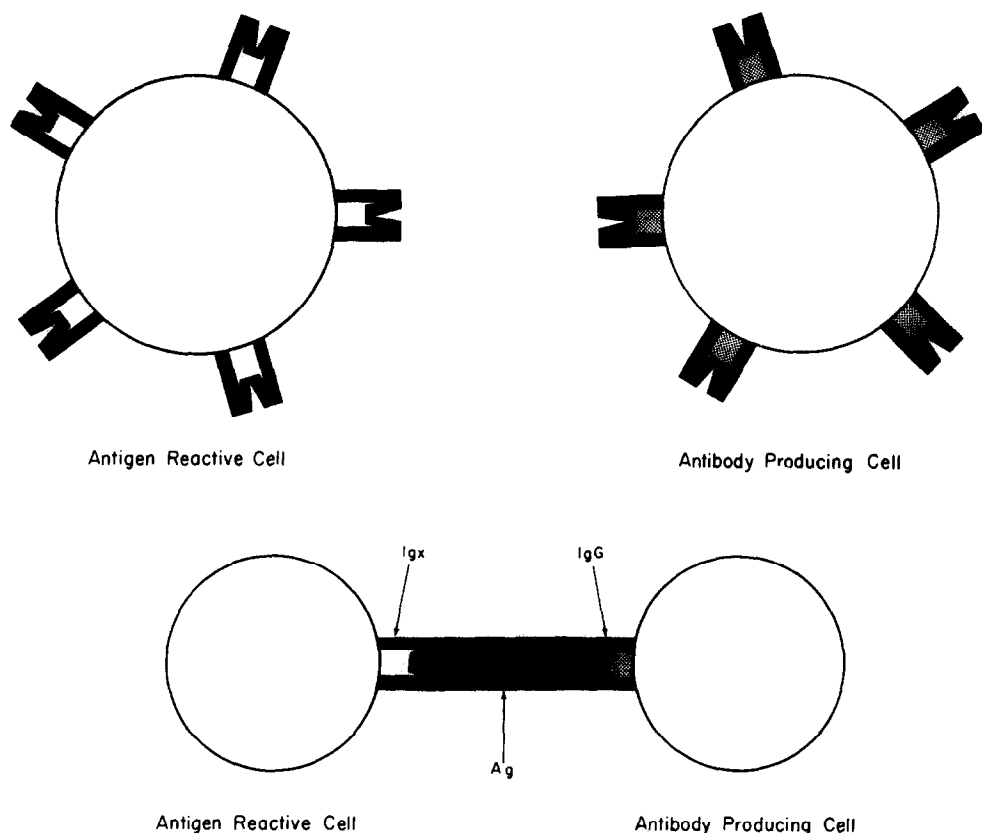


FIG. 3. Concept of antibody formation mediated by cell interaction. Ag, antigen; IgX, recognition unit of the antigen reactive cell; IgG, residual antibody bound to antibody-producing cell.

and bivalent (Fab_2') fragment of ALS induce blast transformation, but the monovalent (Fab) fragment of ALS is not stimulatory. Francis and Paul¹⁶ and Oppenheim and associates,¹⁷ studying antigen-induced transformation, demonstrated that monovalent haptens (DNP-lysine and orthanilic acid) did not induce transformation. Successful *in vitro* transformation occurred when lymphocytes from sensitized animals were challenged with the appropriate multivalent antigen. Such observations indicate that a cross-linking or bridging of receptors on the lymphocyte surface is necessary to trigger lymphocyte transformation. The monovalent nature of antigen D has been reported previously in relationship to precipitin analysis and passive cutaneous anaphylaxis in guinea pigs.⁶ The failure of antigen D stimulation of lymphocytes obtained from untreated and WST-treated patients also indicates its monovalent character, i. e., it cannot bridge across receptor sites to induce *in vitro* transformation.

Current theories indicate that antibody formation requires the interaction of at least two types of cells: antibody-producing cells and antigen-recognizing or -reactive cells.¹⁸⁻²¹ The recognition units of the antigen-reactive cells are

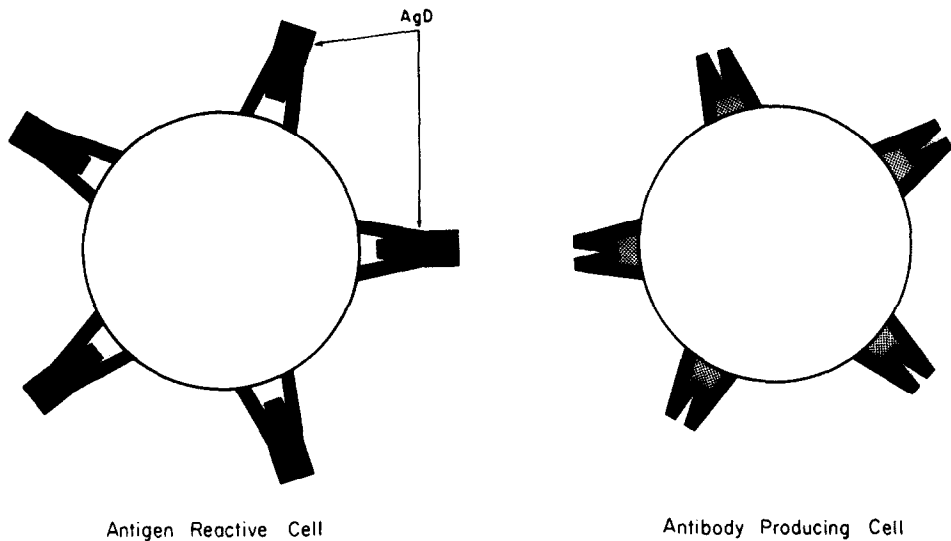


FIG. 4. Proposed site of action of antigen D immunotherapy.

called receptor sites. Although the exact chemical nature of these receptor sites is not known, they are known to have the same antigenic recognition and binding specificities as immunoglobulin molecules. The mechanisms proposed for inducing antibody involve the interaction of the antigen-reactive cell (ARC) and the antibody-producing cell as a consequence of bridging by a bivalent or polyvalent antigen. Fig. 3 presents a simplified picture of the bridging of ARC and antibody-producing cells by a polyvalent allergen (WST).

Attempts to demonstrate antibody (reagin) in culture supernatants in those cultures that had undergone significant levels of transformation were unsuccessful. Thus, in our lymphocyte transformation experiments, we seem not to be observing the proliferation of antibody-producing cells. Grey and associates²² demonstrated that, in rabbits immunized with red blood cells from sheep, the number of transformed lymphocytes was much larger than the number of cells producing antibodies. Shearer and associates²³ showed that antigen-reactive cells exceed antibody-producing cells by a factor of 10 to 1,000 and concluded that in vitro transformation of lymphocytes probably reflects the activity of antigen-reactive cells. Consequently, the lymphocyte transformation observed in our experiments is probably due to the proliferation of antigen-reactive cells; Fig. 4 suggests that the site of action of antigen D is on the ARC. The failure of lymphocytes obtained from patients treated with antigen to respond in vitro to WST strongly suggests that the monovalent antigen D reacting with the receptor sites on the ARC prevents: (1) the reaction of these cells with the polyvalent allergen (WST) and (2) the interaction of the ARC with the antibody-producing cell, thus eventually suppressing antibody formation.

More recent experiments demonstrates that the in vitro response of lymphocytes obtained from antigen D-treated patients is at least partially re-

covered if the patient does not continue monthly treatment. Thus, within 3 months after the termination of antigen D immunotherapy, sufficient antigen D may dissociate from the receptor sites on the ARC to permit the in vitro lymphocyte response of these cells with WST. These studies may have practical therapeutic value, suggesting that patients who have undergone a complete season of antigen D immunotherapy may be maintained with as few as 4 injections per year. Approximately 40 patients are currently being treated with monthly injections of a maintenance dose of antigen D. Cells obtained from these patients show no in vitro response to WST; their clinical symptoms during the coming pollen season will be followed closely.

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