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IMMUNOLOGY OF REAGINIC ALLERGY: *IN VITRO* STUDIES

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SUMMARY

The use of human leucocytes for *in vitro* studies of histamine release is reviewed. Several theoretical aspects and applications of the *in vitro* procedure are discussed. These include sensitivity of the cells to antigen as a reflection of disease severity, *in vitro* assays of reagin and the effect of parenteral immunization on the activity of human reaginic and blocking antibodies.

INTRODUCTION

Recent progress in our understanding of reaginic allergy stems from several lines of investigation; isolation of the etiological agent of the disease, IgE (Johansson & Bennich, 1967; Ishizaka & Ishizaka, 1969), purification and characterization of several important allergens (King & Norman, 1962; King, Norman & Connell, 1964; King, Norman & Lichtenstein, 1967), and the development of *in vitro* methods for studying the mechanism of reaginic hypersensitivity (Osler, Lichtenstein & Levy, 1968). This report will deal with the latter and, more specifically, with the biological activities of the immunoglobulins which participate in the histamine release process. Although the structure of reaginic antibodies is now being elucidated and has been shown to resemble the other classes of immunoglobulins (Bennich & Johansson, 1967; Ishizaka & Ishizaka, 1968) the functional attributes of reaginic antibodies remain somewhat unique. A recent study of the blocking antibodies indicated that more than 95% of this activity was associated with the IgG fraction (Lichtenstein, Holtzman & Burnett, 1968). In view of the complexity and heterogeneity of all immune responses, it may be anticipated that additional studies of individual sera will show that the blocking, and possibly sensitizing, activities will be found in more than a single class of immunoglobulins. Heterogeneity among the skin sensitizing antibodies has been demonstrated for the guinea-pig immunoglobulins (Oliveira *et al.*, 1970) and may well be duplicated in the human situation.

The *in vitro* studies of human reaginic allergy have been based on the initial observations

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of Katz and Cohen in 1941 with blood from ragweed sensitive individuals. These findings were confirmed and extended by Noah, Middleton, Sherman and VanArsdel (reviewed by Osler *et al.*, 1968). On the basis of these studies we undertook a systematic investigation of histamine release from suspensions of washed human leucocytes. Several parameters of this reaction have been described, including the extent of sensitization of the target cell, the antigen requirements and the role of reaginic and blocking antibodies. The relevance of these studies to reaginic allergy is documented below. Since the biological activities of the participating immunoglobulins are demonstrable *in vitro*, many parallelisms have been drawn between the leucocytic response and the clinical disease.

LEUCOCYTE SENSITIVITY AS AN INDEX OF DISEASE SEVERITY

Leucocytes from allergic individuals vary greatly in their response to ragweed pollen antigen as judged by the *in vitro* release of histamine. At one end of the spectrum are the cells of non-allergic individuals which fail to release detectable amounts of this vasoactive amine on challenge with as much as 10 μg of the purified antigenic protein. At the other extreme are the cells of pollen-sensitive patients whose leucocytes release 50% of the cellular histamine store with 10^{-6} μg or less of the same antigen. An indication of these dose–response relation-

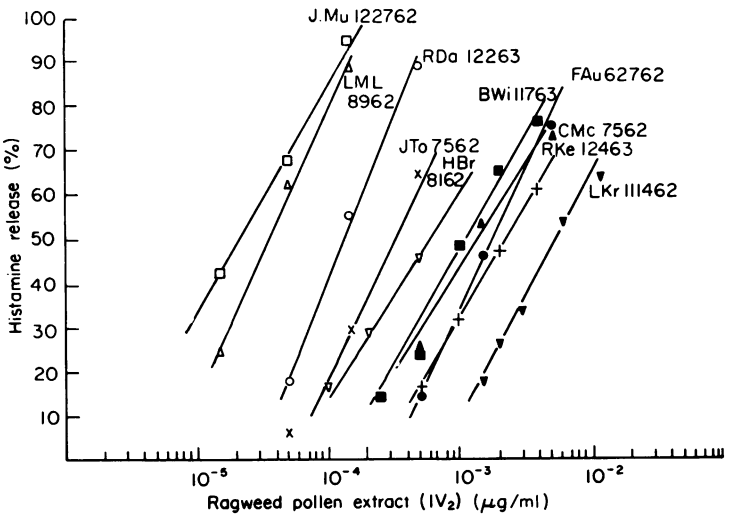

FIG. 1. Dose–response relationships for histamine release as a function of antigen concentration (cells from ten ragweed sensitive donors). (Reproduced from Lichtenstein & Osler, 1964, by kind permission of the Editor.)

ships is shown in Fig. 1. The pertinence of these observations to the clinical situation has been established in several studies during the past 5 years in which the leucocyte response to pollen antigen was compared to the disease severity. The results of one such study are depicted in Fig. 2 and an overall evaluation of this work is given by Lichtenstein & Norman (1969) These studies have led to the conclusion that the antigenic release of histamine from human leucocytes generally reflects the severity of ragweed hayfever when objective methods for evaluating the latter are available.

LEUCOCYTE SENSITIVITY AS A CONSEQUENCE OF REAGIN FIXATION

The evidence accumulated during the past few years establishes that the immune release of histamine from human leucocytes is attributable to their burden of reaginic antibody. As noted above, the correlation of symptom severity with the extent of the *in vitro* reaction is consistent with the notion that the leucocyte response is due to the etiological agent of the disease, IgE. Studies with Dr Levy have shown that leucocytes furnished by non-allergic donors may be sensitized passively under *in vitro* conditions (Levy & Osler, 1966, 1967). Passive transfer is achieved by incubating non-reactive blood leucocytes with serum from an allergic donor. After washing, these cells release histamine on interaction with ragweed pollen antigen in a serum-free medium. There is now little doubt that reaginic antibody mediates the *in vitro* reaction. Thus, passive leucocyte sensitization succeeds only with sera

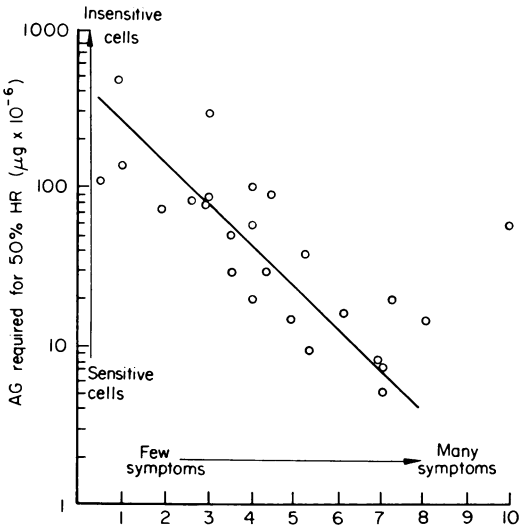

FIG. 2. The relation between *in vitro* leucocyte sensitivity as judged by duplicate titrations performed in March and April 1964 and the symptoms these individuals suffered during the ragweed season of the same year. The patients evaluated their symptoms twice daily. Spearman rank correlation coefficient for patient evaluation = 0·765 $P > 0{\cdot}01$. AG, Antigen; HR, histamine release. (Reproduced from Lichtenstein *et al.*, 1966, by kind permission of the Editor.)

from allergic individuals. The cellular response is immunologically specific and is abolished by prior heating of the serum. The *in vitro* sensitizing capacity of a reaginic serum is well correlated with its ability to transfer a Prausnitz–Küstner (P–K) reaction. Studies with antisera to IgE have also demonstrated that a greater quantity of reaginic antibody is present on leucocytes furnished by ragweed sensitive donors than by non-allergic individuals (Lichtenstein, 1968), a finding consistent with higher serum levels in patients with allergic disease (Johansson & Bennich, 1967). Finally, passive sensitization *in vitro* renders the cells more sensitive to the histamine-releasing action of an antiserum to IgE (Ishizaka & Ishizaka, 1968).

The mechanism of the cell–antibody reaction which results in leucocyte sensitization

remains obscure, since little is known about the nature of the cell receptor site or the structural features of IgE which are essential for this process. The available information suggests that the mediating immunoglobulin undergoes conformational changes during the process of cell fixation as well as in the sequence leading to histamine release. With respect to the sensitization process, it has long been known that heating or reduction and alkylation of reaginic sera destroys their capacity for passive sensitization, possibly through alteration of the disulphide bonds in the Fc fragments. With respect to the release process, it has also been shown that immune complexes formed on the cell surface with an excess of antigen release histamine inefficiently (Osler *et al.*, 1968).

The binding of reaginic immunoglobulins to human cells has been studied indirectly, through the ability of the treated cells to release histamine (Levy & Osler, 1966, 1967). At least two limitations are apparent in this procedure. Only about 20% of non-allergic

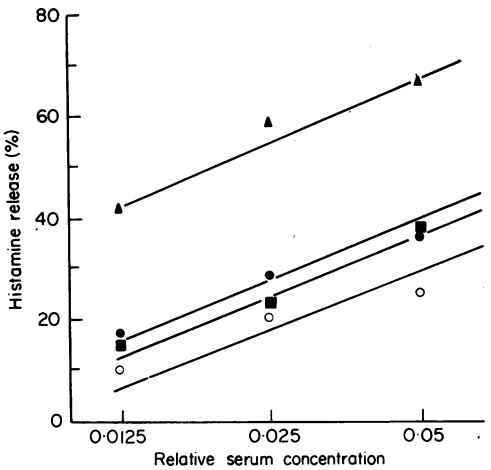

FIG. 3. Passive sensitization of human leucocytes *in vitro*: effect of heparin 10 μg/ml, EDTA 4×10^{-3}M, and a combination of the two on serum dose–response curves. (Experiment 121465: cells, JoGa; histamine available, 0·11 μg/7×10^6 white blood cells; serum DiGu 111665; sensitization 120 min at 37°C; ragweed pollen antigen E, 1×10^{-2} μg/ml). ▲, Heparin + EDTA; ■, heparin; ●, EDTA; ○, buffer. (Reproduced from Levy & Osler, 1967, by kind permission of the Editor.)

donors furnish cells capable of sensitization. Moreover, the binding of reagins to cells lacking histamine (e.g. lymphocytes) is not registered in this reaction. These difficulties notwithstanding, several parameters of the sensitization process have been identified. Cell sensitization, *in vitro*, proceeds best under physiological conditions of pH (7·4) and temperature (37°C). The time required for incubating cells with allergic serum so to obtain a 50% response at 37°, 20° and 4°C is approximately 15, 40 and 120 min, respectively, suggesting that simple adsorption is not an adequate explanation for this reaction.

It would appear that proper steric relationships must be satisfied for optimal cell–reagin interaction. This conclusion is based on the changes observed in cell sensitization by the incorporation of negatively charged compounds like heparin and EDTA. Each of these anionic substances enhances *in vitro* sensitization as measured in terms of histamine release. Chelation of divalent cations does not seem to explain the action of heparin and EDTA.

Heparin is by far the weaker chelating agent, yet it exerts a pronounced synergistic effect on the action of EDTA (Fig. 3). The suggestion is offered that these polyvalent anions provide a more suitable region of complementarity for positively charged groups on the immunoglobulin, thereby resulting in firmer cell binding. It is also of interest in this connection that preincubation of ragweed sensitive cells with calcium and, to a lesser extent, with magnesium reduces the subsequent response to antigen.

IDENTIFICATION AND FATE OF THE TARGET CELL

There is little doubt that the blood basophil which constitutes less than 1% of the total leucocyte population, is the major source of the cellular histamine liberated in the reaction with antigen. As discussed earlier (Osler *et al.*, 1968), there is reason to believe that the neutrophilic granulocytes also participate in the allergic response. This supposition assumes considerable importance in the interpretation of studies bearing on the mechanism of immune histamine release. If the neutrophilic granulocytes do indeed take part in the wheal and flare response, it may be concluded that the antigenic release of histamine does not lead to irreversible damage of the target cell.

The admixture of antigen with reagin-bearing cells initiates a sequence of reactions at the cell membrane which culminates in the cellular release process. The initial reactions can be interrupted by lowering the reaction temperature or removing the divalent cations which are essential for the cellular response (Osler *et al.*, 1968). Restoration of the optimal environmental conditions renews the process. Inhibitors of cellular metabolism block immune histamine release. Thus, Lichtenstein has shown that inhibitors of glycolysis, but not of oxidative metabolism, block the release of histamine (Lichtenstein, 1968). He has also reported that theophylline, an inhibitor of a phosphodiesterase which inactivates 3′,5′ cyclic AMP, also inhibits immune histamine release (Lichtenstein & Margolis, 1968). Catecholamines which increase intracellular AMP, likewise block histamine release at an antigen-activated stage. These interesting findings provide further indication that only a physiologically active cell is capable of mediating this allergic response. The recent report by Levy and Carlton to the effect that colchicine inhibits the allergic release of histamine provides additional evidence in this regard (Levy & Carlton, 1969).

Currently we view the allergic release of histamine as a heightened secretory response triggered by IgE on the cell surface in its reaction with antigen. Since the entire cellular store of histamine may be liberated without a detectable enhancement of intracellular potassium flux, it would seem that cytotoxicity is not a necessary concomitant of the *in vitro* response.

INFLUENCE OF PARENTERAL THERAPY ON THE *IN VITRO* LEUCOCYTE RESPONSE

Blocking antibody levels

The estimation of blocking antibody activity has proven to be a useful feature of the *in vitro* leucocyte response. This assay is based on the finding that serum antibody can combine with the antigen in the fluid phase of reaction mixtures and deflect it from the histamine-laden target cell. As seen in Fig. 4 the substitution of allergic human serum for that obtained from a non-ragweed sensitive individual, shifts the dose–response curve

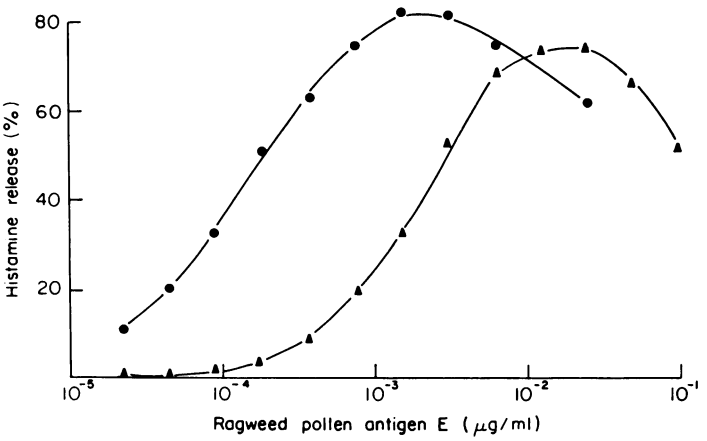

FIG. 4. Histamine release from ragweed-sensitive leucocytes by ragweed antigen E in the presence of normal human (●) and allergic human serum (▲), both at a final dilution of 1 : 10. Cells, RaDa; serum, DeGu. (Reproduced from Osler *et al.*, 1968, by kind permission of the Editor.)

towards higher antigen levels. For the data in Fig. 4, the presence of allergic serum produces a ten-fold increase in the amount of antigen required to obtain a 50% response. The blocking activity is expressed as a ratio of antigen requirements for the test and normal sera, as illustrated in Fig. 5. Blocking activity has been detected in the sera of all untreated ragweed sensitive patients (Lichtenstein & Osler, 1966) suggesting that serum reagins may also protect the target cells from antigenic histamine release. The sera of untreated ragweed sensitive patients bind about 60% of the antigen in the fluid phase.

Following parenteral immunotherapy, the antigen binding capacity of the serum increases

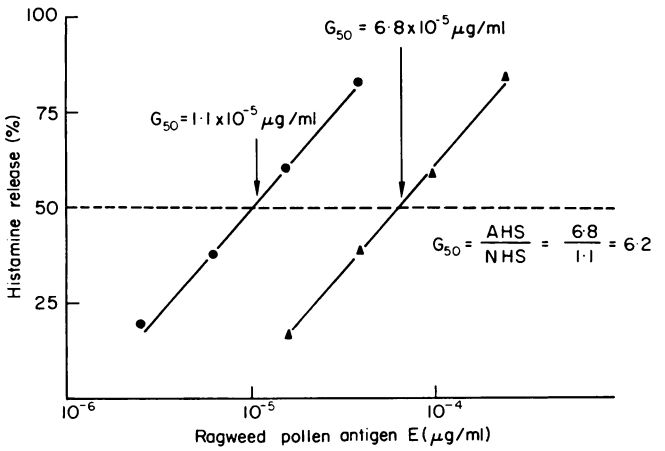

FIG. 5. Dose–response curves of one individual's cells in 10% normal (NHS, ●) and autologous allergic (AHS, ▲) human serum. The method of calculating the level of antibody activity, or the G_{50} (AHS/NHS), is indicated. Cells and serum LML. (Reproduced from Osler *et al.*, 1968, by kind permission of the Editor.)

readily, the rise being proportional to the intensity, and schedule of immunization. The data in Fig. 6 depict the changes in blocking antibody activity in the sera of three patients as a function of preseasonal immunization. As indicated, most of this binding activity has been localized to the IgG fraction of the sera.

Moreover, these immunization regimens have been associated with favourable therapeutic results (Lichtenstein *et al.*, 1966; Lichtenstein & Norman, 1969), and marked increases in serum blocking activity as typified in Fig. 7. A causal relationship between serum blocking

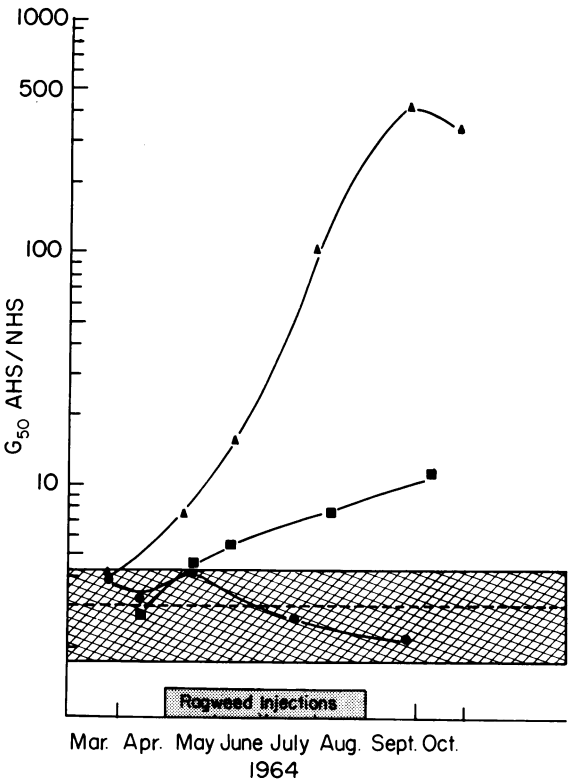

FIG. 6. Analyses of the antigen-neutralizing capacity of serum specimens from three individuals treated as indicated. The symbol G_{50} AHS/NHS refers to the quantity of ragweed pollen antigen (IV_2) required for 50% histamine release in allergic human serum (AHS) divided by that quantity needed for the same response in the presence of normal human serum (NHS). Cross-hatched area represents average G_{50} AHS/NHS of non-treated patients ± 1 SD. ▲, Treated with ragweed antigen (IVc); ■, treated with crude ragweed extract; ●, placebo treatment. (Reproduced from Lichtenstein & Osler, 1966, by kind permission of the Editor.)

antibody levels and symptomatic improvement has been difficult to establish unequivocally, and indeed, is still a matter of some dispute. From an immunological viewpoint several considerations may be advanced to account for this uncertainty. The severity of the clinical disease represents a summation of many factors, including some which have no immunological basis. Most of the laboratory studies based on the *in vitro* leucocyte response have dealt with antigen E. The response to a single antigenic determinant is usually complex and heterogeneous so that even studies with one antigen may not reflect the entire clinical picture.

Another consideration is referable to the nature of the blocking immunoglobulin and its site of action. There is reason to believe that the events taking place on the mucous membranes of the upper respiratory tract may be far more indicative of clinical disease severity than those presumed to occur with the serum reactants. Further, the recognition that secretory immunoglobulins may play an important role in pollinosis deserves much further study. Finally, parenteral immunotherapy also reduces serum reagin levels so that clinical improvement need not be due entirely to the blocking antibodies.

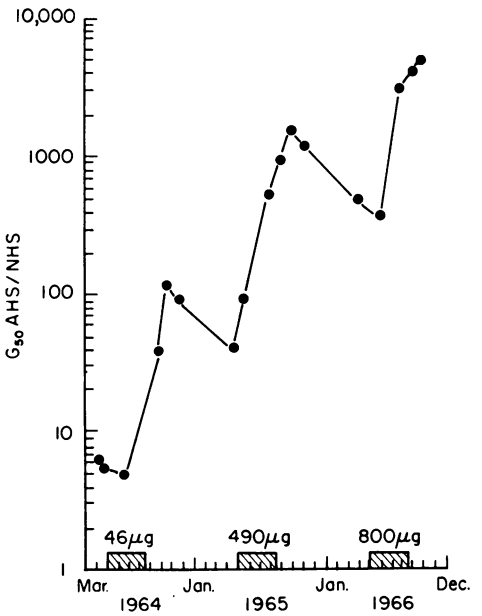

FIG. 7. Variations in antigen neutralizing capacity ('blocking' antibody) in the serum of one individual following therapeutic immunization over a 3-year period. (Reproduced from Osler *et al.*, 1968, by kind permission of the Editor.)

Serum reagin levels

As shown by Johansson, serum IgND levels (the myeloma counterpart to IgE) rise from about 50 to 1800 ng/ml (Johansson, 1968). Until recently, an accurate assay for such low levels of reagin was not feasible. Even the present methods may not indicate the antigenic specificity of these immunoglobulins. The *in vitro* histamine release studies, on the other hand, describe the biological action of reaginic antibodies to the test antigen, yielding activity but not weight measurements (Osler *et al.*, 1968). In studies with Dr Levy, an *in vitro* assay for reagins was developed which is well correlated with Prausnitz–Küstner reactions (Levy & Osler, 1967). Application of this assay to clinical situations further elucidated some of the immunological events in human reaginic allergy. As typified in Fig. 8, annual exposure to allergens increases serum reagin titres which, in the case of ragweed pollen, wane during the winter and spring seasons (Levy & Osler, 1967). Interestingly, parenteral immunotherapy suppresses the annual increase in serum reagin activity (Fig. 9).

It follows therefore that injections of pollen antigen serve a dual purpose. Not only is the blocking antibody activity increased, but reaginic immunoglobulins, the etiological agents of

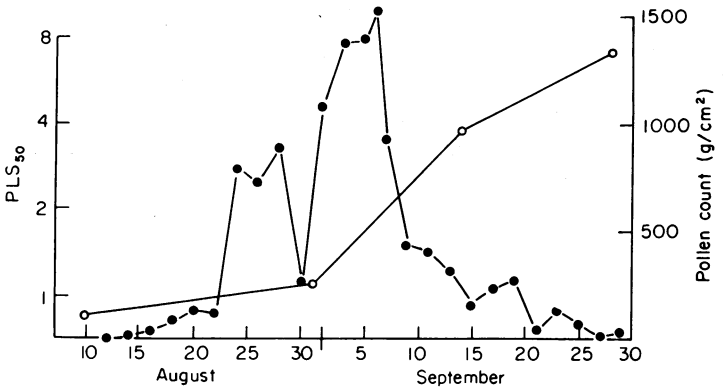

FIG. 8. PLS_{50} titres (○) of serum EdSo during 1966 ragweed pollen season. Experiment 101766. Cells, JoRu; sensitization, 120 min at 37°C. PLS_{50} refers to the serum dilution which sensitizes normal cells for a 50% histamine response to antigen. ●, Pollen count. (Reproduced from Osler *et al.*, 1968, by kind permission of the Editor.)

ragweed hayfever, are prevented from attaining their usual levels. These laboratory findings have received encouraging support from recent clinical studies with children (Sadan *et al.*, 1969; Levy, Goldstein & Lichtenstein, 1969). Children treated with aqueous ragweed extract showed marked clinical improvement. This alleviation of disease severity was accompanied by an average rise of twenty-fold in serum blocking activity and with a diminished histamine

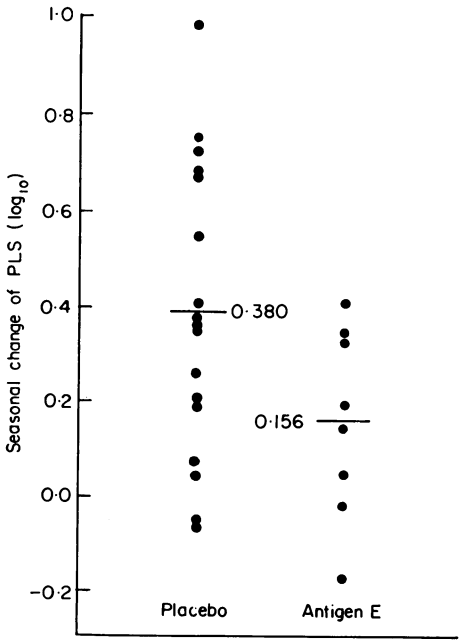

FIG. 9. Range of increase in PLS_{50} titres. Ordinate: $\log_{10}$ post-season PLS titre–$\log_{10}$ pre-season PLS titre. Placebo: twelve patients in seventeen pollen seasons of 1964–66. Antigen E; eight patients in pollen season of 1966, $0{\cdot}05 > P > 0{\cdot}025$ for difference between the means, Student's *t*-test. (Reproduced from Osler *et al.*, 1968, by kind permission of the Editor.)

response by the treated patients' leucocytes. In contrast, the cells furnished by children in the untreated group showed unchanged or greater responses to antigen. Serum reagin levels were diminished slightly, if at all. Since the decreased cell responses noted in this study may also be considered a result of curtailed reagin synthesis and/or fixation, it would appear that immunotherapy produced favourable results in this group of children, as was previously recorded for adults (Lichtenstein & Norman, 1969). The results obtained in these combined clinical and laboratory studies seem to justify application of the *in vitro* histamine release procedures to other diseases in which allergic reactions of the immediate type constitute an important facet of the clinical disease.

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