Immunology, 1972, **22**, 1013.

Induction of IgE-Mediated Immediate Hypersensitivity to Group I Rye Grass Pollen Allergen and Allergoids in Non-Allergic Man

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(*Received 29th October* 1971)

Summary. The major (Group I) allergen of rye grass pollen and two of its allergoids, adsorbed on alumina gel, were injected into three groups of non-allergic humans. In addition to inducing the anticipated blocking antibody (IgG) response, all individuals developed immediate skin hypersensitivity to the allergen and its allergoids characteristic of reaginic antibody-(IgE-)mediated reactions. At some time during the course of the study, virtually every individual's peripheral blood leucocytes were also found to release histamine when challenged *in vitro* with low concentrations of allergen and allergoids. Quantitatively, each person's skin and leucocyte sensitivities were not as well correlated as in naturally allergic people. Leucocyte responsiveness was generally shortlived, but could be restored by antigenic restimulation. Allergoid:allergen sensitivity ratios were greater in allergen- than allergoid-immunized individuals, but less than in naturally allergic individuals. Unexpectedly, allergoid-immunized individuals' leucocytes were more sensitive to allergen than allergoid. Despite the observed skin and leucocyte reactivities, none of the people showed clinical manifestations of hay fever following natural exposure to pollen.

The skin sensitivity of the artificially sensitized individuals could be passively transferred to non-allergic humans by intradermal injection of serum (P-K Test), thereby implicating the involvement of IgE antibody. Further proof of the role of IgE was obtained by blocking the P-K test, either by heating the serum or by adsorbing it using an anti-IgE immunosorbent.

INTRODUCTION

The rye Group I component is the major allergen of rye grass pollen (Johnson and Marsh, 1965; Marsh, Milner and Johnson, 1966). Group I allergens, immunochemically related to rye Group I, are found in many other grass pollens causing hay fever in allergic man (Marsh, Haddad and Campbell, 1970; Marsh and Campbell, unpublished).

By mild formalin treatment, the rye Group I allergen may be converted into an 'allergoid'—briefly defined as a modified allergen of greatly reduced allergenic reactivity, but for which other antigenic properties characteristic of the native molecule are largely

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retained (Marsh, Lichtenstein and Campbell, 1970). Allergoid derivatives of Group I may be prepared by treating the allergen with formaldehyde solution alone, yielding the so-called 'normal-allergoid', or by treating the allergen with formaldehyde together with the amino acid lysine, which becomes chemically incorporated into the resultant 'lysine-allergoid'. Group I allergoids are almost as effective as native Group I in stimulating guinea-pigs to produce 'blocking antibody', i.e. antibody which is able to neutralize *in vitro* the allergenic reactivity of the native allergen in the allergic human leucocyte system (Lichtenstein, Norman, Winkenwerder and Osler, 1966; Marsh, Lichtenstein, and Campbell, 1970).

Allergoids have considerable potential for use in the immunotherapy of allergic man. But, prior to conducting a controlled clinical trial, we needed to compare blocking antibody production by allergoids and allergen in man. Therefore, non-allergic human volunteers were immunized with the native allergen and its allergoids. As expected, production of allergen-neutralizing (blocking) antibody against both the allergen and its allergoids was observed: these results have briefly been described (Marsh, 1971a) and will form the subject of a subsequent paper. However, we also observed the concomitant development of skin and leucocyte reactivity, characteristic of reaginic hypersensitivity, toward both the allergen and allergoids. The development and characteristics of this artificially induced hypersensitive state form the basis of the present report.

MATERIALS AND METHODS

Antigens

Group I allergen was prepared from perennial rye grass pollen according to the procedure of Johnson and Marsh (1965). Its 'normal-allergoid' was prepared by incubating the allergen with dilute formaldehyde solution for 32 days at 32°; its 'lysine-allergoid' was produced under similar conditions by incubating the allergen with formaldehyde and lysine (Marsh, Lichtenstein and Campbell, 1970). The allergen and allergoids used for the present study were the same or similar preparations to those used previously for guinea-pig immunization studies (Marsh, Lichtenstein and Campbell, 1970). The residual allergenicity of such allergoids, relative to the native allergen, was found to range between about 0·7 per cent and 0·0006 per cent (G.M. = 0·08 per cent).

Non-allergic humans

Three groups of five adults (aged 25–45 years) gave fully informed consent to be immunized with the allergen (Group A), normal-allergoid (Group B) and lysine-allergoid (Group C). All individuals were clinically non-allergic to common allergens, including grass pollens, with the exception of one individual who reported mild sensitivity to house dust. However, this person was later negative by scratch test with a potent house dust extract at 250,000 PNU/ml and gave only a 1+ (probably toxic) intradermal skin test to 0·05 ml of the dust extract at 1000 PNU/ml. Before immunization, all subjects were negative by intradermal skin test using 0·05 ml of crude grass pollen extract at 10,000 PNU/ml.

Immunization schedule

The antigens were completely adsorbed on a suspension of alumina gel (Type Cγ: Chase, 1967) in buffered physiological saline at pH 7·8, yielding a final suspension containing 100 μg antigen and 4·5 mg $Al(OH)_3$ per ml. The subjects were immunized sub-

cutaneously according to the schedule shown in Table 1. The antigen dosage used for the last injection at 63 weeks was determined by the degree of the person's immediate and delayed skin reactivity to the immunizing antigen at that time. Any immediate (15 minutes) and delayed (24–48 hour) reactions to the injections were recorded.

TABLE 1

SCHEDULE OF IMMUNIZATIONS

Date (day/month/year)	Weeks	Group A Allergen (μg)	Groups B,C Allergoid (μg)	Alumina adjuvant
12/5 to 18/6/69	0–5		Grass pollen season*	
13/5/69	0	100	100	Yes
20/5/69	1	100	100	Yes
27/5/69	2	100	100	Yes
30/6 to 8/7/69	7–8	100	100†	Yes
4/11/69	25	100	100	No
7/1 to 13/1/70	34–35	—	50	No
8/5 to 30/6/70	52–59		Grass pollen season*	
28/7/70	63	1;10‡	10;50;100§	No
28/4 to 29/6/71	102–111		Grass pollen season*	

* Period during which counts on Rotoslide Sampler were greater than 10 grains/cm.^{2}
† Subject W.T. immunized with normal allergoid (Group B see Table 2) missed the injection at 7–8 weeks. He was subsequently injected with 200 μg antigen in aqueous solution at 34 weeks rather than with the 50 μg dose which other allergoid-immunized individuals received.
‡ Subject F.A. received 1 μg, subject J.S. was not injected and subject M.W. received 10 μg allergen.
§ Subject D.M. received 10 μg, subject J.Mc C. received 50 μg and all other allergoid immunized people received 100 μg antigen.

Toxoid-alumina suspensions similar to the materials used for this study are commonly used in prophylactic immunization of man against bacterial toxins. Furthermore, the quantities of antigen administered here were similar to those generally given to allergic individuals as therapy for their disease, and much higher total doses of purified ragweed Antigen E have been administered to ragweed-allergic individuals without ill-effects (Norman, Winkenwerder and Lichtenstein, 1971).

The 1969, 1970 and 1971 Baltimore grass pollen seasons occurred during the period of our study (Table 1). Maximal pollen counts in the range of about 150–400 grains per cm^2 on the Intermittent Rotoslide Sampler (Ogden and Raynor, 1967) were observed at 1–4 weeks, 52–56 weeks and 106–110 weeks.

During the early part (6–30 weeks) of the above immunization schedule, a total of six individuals (two from each group) dropped out for reasons given in the footnote to Table 2. In addition, at 30 weeks, subject J.S. in Group A discontinued with the immunization and skin testing due to mild discomfort from local skin reaction following immunization; however, she donated blood samples periodically.

Allergenic reactivity

Direct skin tests. Repeated skin tests were performed 25–99½ weeks after the first immunization using intradermal injections (0·05 ml) of solutions of all three antigens in each person. Since skin sensitivity varied widely with respect to time, person and immunizing antigen, we used antigen concentrations ranging between 100 μg/ml and 10^{-6} μg/ml, as appropriate. Tris-buffered saline, pH 7·3, containing 0·03 per cent human serum albumin

TABLE 2

ONSET OF SKIN REACTIVITY AND LEUCOCYTE RESPONSIVENESS TO IMMUNIZING ANTIGENS

				Skin reactions to immunizing antigens (100 μg/ml before 5th injection (25 weeks)		Leucocyte responsiveness to immunizing antigens at 29½–30½ weeks	
	Subject	Sex	Race	Immediate	Delayed	Sensitivity, $S_{20\ per\ cent}$ (μg/ml)	Max reactivity, R_{max} (per cent)
Group A (immunized with native allergen)	F.A.	F	C	2+	2+	$4{\cdot}0\times10^{-4}$	100
	F.B.*	M	C	Dropped out		Dropped out	
	R.G.†	M	C	Dropped out		Dropped out	
	J.S.	F	C	2+	3+	$4{\cdot}6\times10^{-3}$	66
	M.W.	F	C	4+	3+	$5{\cdot}0\times10^{-3}$	19
Group B (immunized with normal-allergoid)	J.McC.	F	C	±	2+	0	0
	M.M.‡	F	N	Dropped out		Dropped out	
	D.S.†	F	C	Dropped out		Dropped out	
	W.T.	M	N	0	0	0	0
	A.W.	M	N	3+	0	$1{\cdot}5\times10^{-2}$	33
Group C (immunized with lysine-allergoid)	M.B.§	F	C	0	0	Dropped out	
	G.F.§	M	C	0	0	Dropped out	
	D.M.	M	C	2+	±	$2{\cdot}4\times10^{-2}$	36
	T.W.	M	N	0	±	$6{\cdot}4\times10^{-2}$	22
	J.M.W.	M	C	2+	0	0	6

Drop outs:
* Had marked (4+)delayed reaction following the 4th injection (7–8 weeks).
† Dropped out between 3rd and 4th injections due to illness and pregnancy respectively; no skin reactions observed.
‡ Dropped out between 4th and 5th injections due to moving from Baltimore area; no skin reactions observed.
§ Dropped out soon after 5th injection due to moving from area and lack of interest in continuing with study; no skin reactions observed.
Grading of skin reactions (0·05 ml *injected*):
Immediate:
± :Wheal dia., 0·5–1·0 cm with no pseudopods; erythema dia., 0·5–1·0 cm.
1+ :Wheal dia., 0·5–1·0 cm with no pseudopods; erythema dia., 1·0–2·0 cm.
2+ :Wheal dia., 0·5–1·0 cm with no pseudopods; erythema dia., 2·0–3·0 cm.
3+ :Wheal dia., 1·0–1·5 cm with at least 1 pseudopod; erythema dia., 3·0–4·0 cm.
4+ :Wheal dia., > 1·5 cm with multiple pseudopodia; erythema dia., > 4·0 cm.
Delayed: dia. of swelling: ± : 0·5–1·5cm.
1+ : 1·5–3·0cm.
2+ : 3·0–4·5cm.
3+ : 4·5–6·0cm.
4+ : > 6·0cm.

as a stabilizer, was used as the diluent. Wheal and erythema reactions were measured 15 minutes after injection and the antigen concentrations giving a 2+ skin reaction (5–10 mm diameter wheal without pseudopods; 20–30 mm diameter erythema) were recorded or interpolated from the lesser and greater reactions at two antigen concentrations differing by ten-fold. The antigen concentrations yielding 2+ reactions on the same individual at different times were plotted on a logarithmic scale.

Passive transfer tests. Sera from three highly skin sensitive individuals (one from each immunized group), taken at times of maximal leucocyte and/or skin sensitivity, were used in passive transfer experiments of the Prauznitz-Küstner (P-K) type. For one of these donors (F.A. from Group A), sera taken at ten different times during the course of the study were used to follow the variation in the titre of passively transferable reaginic antibody with time. The selected donors had no history of hepatitis or blood transfusions. Australia antigen tests on the sera were also negative. Sterile dilutions of the sera in the range 1:5 to 1:640 were prepared in physiological tris-saline buffer at pH 7·5, 0·03 per cent human serum albumin additionally being incorporated throughout the range 1:80 to 1:640. Samples of the 1:5 dilutions were heated at 56° for 4 hours in order to inactivate any IgE.

The above serum dilutions (0·1 ml), together with tris-saline albumin buffer and 1:5 dilutions of the individuals' pre-immunization (0 week) serum as controls, were injected intradermally into the backs of three non-allergic skin test negative human volunteers. Forty-eight hours later the passively sensitized sites were challenged with the native Group I allergen (0·05 ml at a concentration of 1 μg/ml). Wheal and erythema skin reactions were measured 15 minutes later.

*Testing with sera adsorbed with anti-IgE**: In order to test whether the passively transferable reaginic antibody was of the IgE class, high-titre sera (2·0 ml) from two of the immunized individuals which had previously been tested, were adsorbed overnight at 4° with an anti-IgE immunosorbent suspension (0·5 ml). The immunosorbent had been prepared by coupling the IgG fraction of rabbit anti-human myeloma IgE (rendered ε-chain specific by adsorption) to CNBr-activated agarose beads, according to the method of Porath, Axen and Ernback (1967). One millilitre of this immunosorbent was capable of adsorbing at least 8000 ng of normal IgE. Levels of total IgE were determined on non-adsorbed and adsorbed sera using the radioimmunoassay procedure of Ishizaka, Tomioka and Ishizaka (1970), which is able to detect as little as 5 ng IgE/ml.

Following sterile filtration, the adsorbed sera, and appropriate controls of non-adsorbed sera and pre-treatment sera, were used in passive transfer experiments as described above.

Leucocyte assays

Blood samples were taken from the immunized volunteers at a series of occasions 29½–115 weeks after the initial immunization. Washed leucocytes isolated from these blood samples were used for quantitative assay of the allergenic reactivities of native rye Group I allergen and its allergoids using a micro-modification (May, Lyman, Alberto and Cheng, 1970) of the leucocyte histamine release assay of Lichtenstein and Osler (1964).

Since the percentages of the total cellular histamine released by these antigens was often less than 50 per cent, we chose to express cellular responsiveness in terms of both the antigen concentration required to release 20 per cent of the cells' histamine (cellular sensitivity, $S_{20\ per\ cent}$) and the maximum percentage of histamine released at optimal antigen con-

* The adsorption experiments and IgE determinations necessary for this part of the study were performed by Dr K. Ishizaka.

centration (cellular reactivity, R_{max}). The definitions used here have previously been discussed by Levy, Lichtenstein, Goldstein and Ishizaka (1971).

The comparative values of $S_{20\ per\ cent}$ and R_{max}, obtained at various times for the same cell donor, were plotted on logarithmic and linear scales respectively. The relative sensitivity of a donor's leucocytes to an allergoid and the allergen was determined from the ratio:

$$\frac{S_{20\ per\ cent},\ allergoid}{S_{20\ per\ cent},\ allergen}.$$

Eye tests

Whole rye grass pollen was defatted by extraction with ether and air dried. Increasing doses of up to several hundred pollen grains were introduced under the lower eye lid. The eye was examined for oedema, watering and reddening 10 minutes after each pollen administration, using the unchallenged eye as the control.

RESULTS

The development of reactions characteristic of immediate hypersensitivity (positive wheal and erythema skin tests and leucocyte responsiveness to allergen and allergoids) occurred most readily in individuals of Group A who had been immunized with the native allergen, although some of the allergoid-immunized individuals (Groups B and C) also became sensitive.

Immediate and delayed reactions to intradermal skin tests using antigen solutions at the highest tested concentration (100 μg/ml) were first observed in most individuals at 25 weeks following the 4×100 μg antigen immunizing doses previously injected at 0, 1, 2, and 7–8 weeks (Table 2). Only subject F.B. of Group A showed any reaction (in his case a marked delayed reaction) to the fourth immunizing injection at 8 weeks. Development of immediate and delayed skin sensitivity in people who were negative to the immunizing injection at 8 weeks, but positive to the skin test at 25 weeks, could have occurred at any time between 8 and 25 weeks.* The skin reactions toward the fifth immunizing dose at 25 weeks generally followed the pattern of the intradermal skin tests performed on the previous day. At subsequent immunizations, both immediate and delayed reactions were generally observed to varying degrees, following the pattern of reactions observed to intradermal skin tests performed at the same time.

Leucocyte responsiveness was first measured at 29½ weeks, when all three remaining allergen-immunized (Group A) and three of the six remaining allergoid-immunized (Groups B and C) volunteers, were found to have appreciable leucocyte sensitivity to the immunizing antigen (Table 2). The degree of leucocyte responsiveness of subject F.A. in Group A to the native allergen (Fig. 1) was comparable with the responsiveness of individuals who are naturally sensitive to grass pollen. However, F.A.'s relative cellular sensitivity to the two allergoids was quite different from that seen in naturally allergic people; for both the allergoids, her sensitivity ratios ($S_{20\ per\ cent}$, allergoid: $S_{20\ per\ cent}$, allergen) were found to be about ten, which is two orders of magnitude less than the ratios usually found in naturally allergic individuals.

* We did not perform skin tests during this period since we had not originally planned to study the development of reaginic antibody-mediated response.

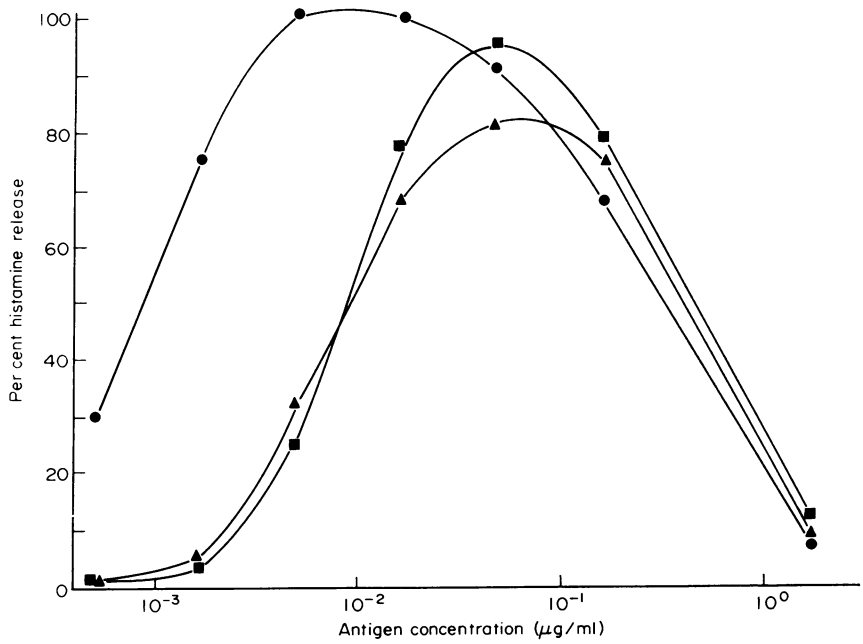

FIG. 1. Antigen-mediated histamine release using isolated leucocytes from subject F.A. in Group A (immunized with native allergen), determined 29½ weeks after the initial immunizing injection. ●, Native allergen ■, normal-allergoid; ▲, lysine-allergoid.

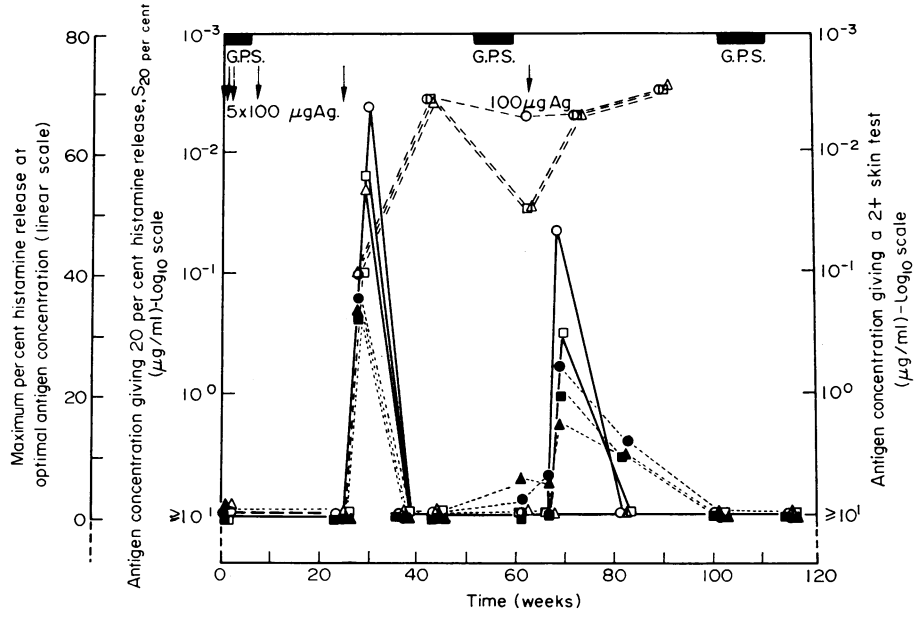

FIG. 2. Time course of leucocyte sensitivity ($S_{20\ per\ cent}$), leucocyte reactivity (maximum per cent histamine released at optimal antigen concentration) and skin sensitivity (antigen concentration giving a 2+ skin test) for subject A.W. in Group B. Immunizations with normal-allergoid were at the times and dosages indicated by the arrows; the grass pollination seasons (G.P.S.) are shown as solid rectangles.

Leucocyte sensitivity, solid line with open symbols; leucocyte reactivity, dotted line with closed symbols; skin sensitivity, dashed line with open symbols. ●, Native allergen; ■, normal-allergoid; ▲, lysine-allergoid

Note: Between the first three sets of symbols (at 0, 25 and 30 weeks), and in other parts of the diagram, triplication of each type of line has been omitted in order to make the diagram clearer.

Fig. 2 illustrates the time course for leucocyte sensitivity, leucocyte reactivity and skin sensitivity to the allergen and its two allergoids for one immunized individual (subject A.W. of Group B). The time course of skin and leucocyte sensitivities to the native allergen *only* have been selected (Fig. 3) to represent our results using all three of the antigens for the nine immunized individuals who remained on the study beyond 30 weeks.

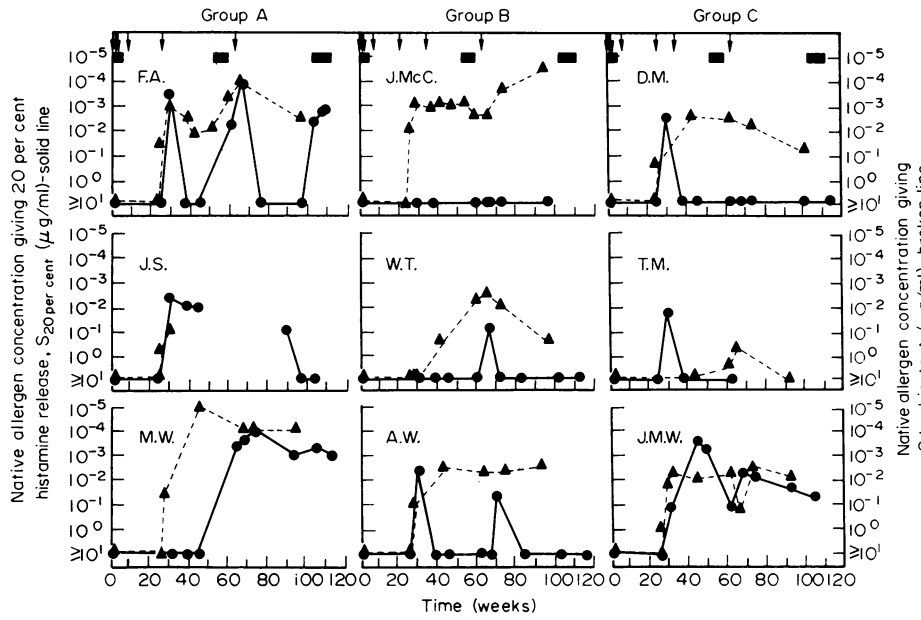

FIG. 3. Time course of leucocyte sensitivity ($S_{20\ per\ cent}$) and skin sensitivity to the *native allergen* for all nine individuals. The times of immunization and the grass pollen seasons are indicated respectively by arrows and solid rectangles at the top of the diagram (for details of immunizing dosages see Table 1). ●, Leucocyte sensitivity; ▲, skin sensitivity.

From all our data, exemplified diagrammatically in Figs 1–3, the following general observations emerged: (1) All individuals developed immediate skin reactivity to the allergen and its allergoids; the sensitivity to allergen usually being the same, or an order of magnitude greater, than to the allergoids; (2) all individuals, except one in Group B (J.McC., Fig. 3), showed leucocyte responsiveness to allergen and allergoids at some time during the course of the experiment; (3) an individual's degree of skin sensitivity to any of the antigens did not correlate well with his degree of leucocyte sensitivity or reactivity at that same occasion; (4) the duration of artificially induced leucocyte responsiveness in non-allergics was usually short-lived (about 2-3 months*), although subsequent antigenic restimulation restored the responsiveness for a similar period; (5) artificially induced skin sensitivity persisted for a much longer period than leucocyte responsiveness; we have not yet observed very marked (over 100-fold) reductions in skin sensitivity for the 40 weeks since the last immunizations; (6) during the course of the study, individuals immunized with the native antigen showed greater leucocyte responsiveness to the immunizing antigen than people immunized with either of the allergoids; (7) as expected, allergen-immunized

* Subjects M.W. (Group A) and J.M.W. (Group C) had more persistent leucocyte responsiveness than other individuals. Their cells' responsiveness was particularly prolonged after the booster injections at 63 weeks, although it slowly diminished in intensity with time.

individuals always showed greater leucocyte responsiveness to the allergen than its allergoids; however, allergoid-immunized individuals almost always showed greater responsiveness to the native allergen than to either of its allergoids*; (8) the leucocyte sensitivity ratios ($S_{20\ per\ cent}$, allergoid: $S_{20\ per\ cent}$, allergen) were nearly all greater for allergen-immunized than for allergoid-immunized individuals; although all such ratios in the present study were lower than we have observed in any naturally allergic individuals; (9) there appeared to be a tendency for allergoid: allergen cell sensitivity ratios to increase as a result of reimmunization for individuals in Group A, but to decrease slightly for individuals in Groups B and C (cf. Fig. 4).

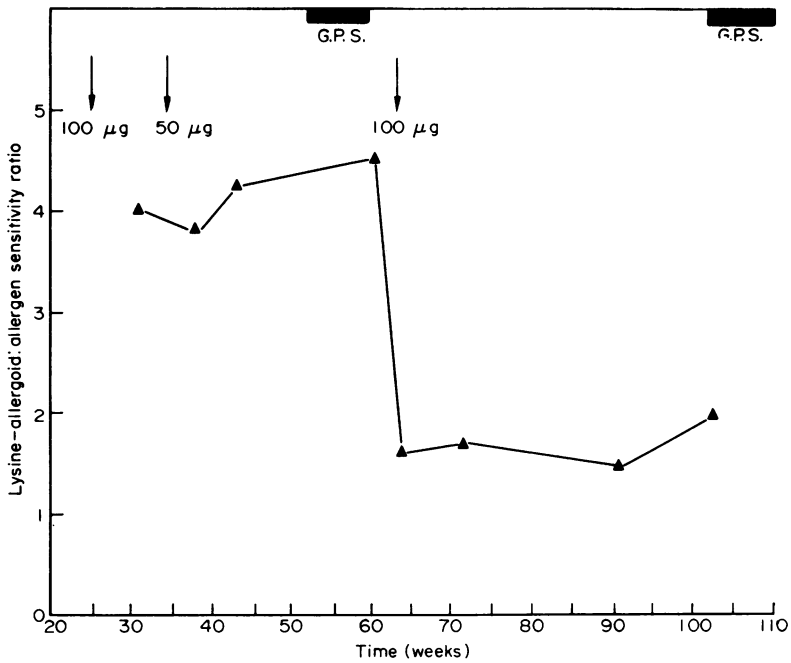

FIG. 4. Variation in lysine-allergoid : allergen leucocyte sensitivity ratio with time for subject J.M.W. of Group C (immunized with lysine-allergoid).

Observations (7) and (8) are illustrated more clearly in Fig. 5 which compares individuals' leucocyte sensitivities ($S_{20\ per\ cent}$) to native allergen with their leucocyte sensitivity ratios to allergoid and allergen. Similar plots for twelve naturally allergic people have been recorded on the same graph. In this figure, all experiments on artificially sensitized people have been recorded wherever their leucocyte histamine release for allergen and allergoids was over 20 per cent. For individuals in Groups A and B and the naturally sensitive people, we have plotted the normal-allergoid: allergen ratios; for individuals in Group C, we have plotted the lysine-allergoid: allergen ratios. The leucocyte sensitivities to allergen of allergen-immunized individuals in Group A were generally similar to those found in naturally highly allergic individuals, but their sensitivity ratios were lower than in naturally allergic people. Allergoid-immunized individuals had lowest sensitivity to allergen and lowest

* On only one occasion in one individual (J.M.W. in Group C) did we find marginally greater leucocyte sensitivity to the normal-allergoid than to the native allergen. We are unable to explain this observation other than its possibly resulting from a technical error.

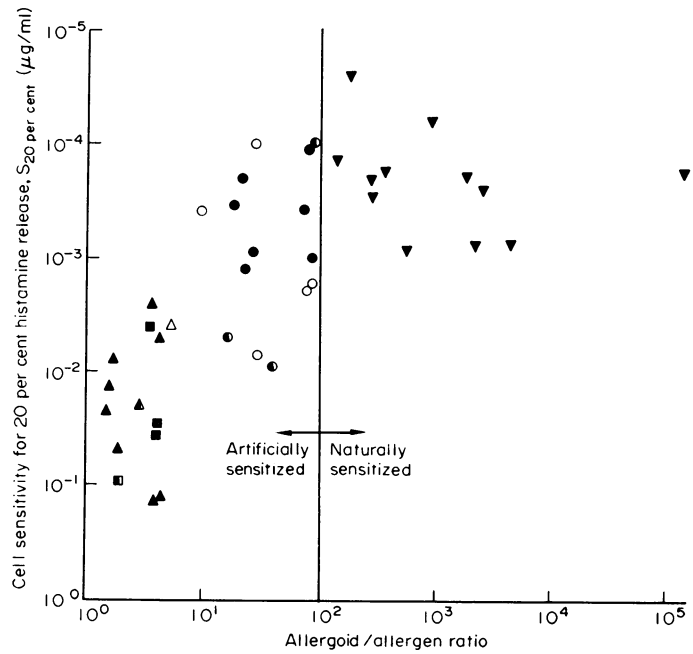

FIG. 5. Comparison of allergoid:allergen leucocyte sensitivity ratios versus leucocyte sensitivity to allergens for both artificially and naturally sensitized individuals.

Artificially sensitized, ○, Group A, F.A.; ◐, J.S.; ●, M.W.; Group B, □, J.McC.; ◧, W.T.; ■, A.W.; Group C, △, D. M.; ◭, T.W.; ▲, J.M.W. ▼, naturally sensitized.

sensitivity ratios. For any of the artificially sensitive individuals, we observed very little difference between the normal-allergoid:allergen and the lysine-allergoid:allergen ratios.

None of the artificially sensitized individuals were found to exhibit symptoms of allergy during the 1970 and 1971 grass pollen seasons. Thus, clinical manifestations of pollen allergy were not correlated with high skin and leucocyte sensitivities to Group I rye allergen, as observed in naturally allergic people (Lichtenstein, Norman and Winkenwerder, 1971). Furthermore, ocular pollen challenge of volunteer F.A. was negative at 29½ weeks, a time when she showed high leucocyte responsiveness (Fig. 1).

In the absence of subcutaneous immunization, at certain times during the course of the study, the leucocytes of two allergen-immunized individuals (F.A. and M.W. in Group A) became dramatically more sensitive to allergen and allergoids (Fig. 3). Specifically, during the periods 42½–60½ weeks and 97½–108 weeks, F.A.'s leucocytes 'turned on'. The 1970 and 1971 grass pollen seasons occurred within these periods of time (Table 1.) However, intradermal skin tests which could possibly have had an immunizing effect were performed as follows: 0·12 μg allergen plus allergoids at 42½ weeks, 0·06 μg at 50½ weeks, 0·001 μg at 97½ weeks, and 0·02 μg at 99 weeks. Subject M.W.'s leucocytes 'turned on' between 43 and 63 weeks, coinciding with the 1970 grass pollen season, and with the added complication of intradermal skin tests, totalling 0·001 μg antigen, at 43½ weeks. No significant boosting of leucocyte sensitivity was seen in the absence of regular subcutaneous immunization in any other individual studied.

Table 3 presents results of experiments where sera from three individuals were passively transferred to the skin of non-allergic human recipients, and the injected sites challenged

TABLE 3

PASSIVE TRANSFER (P-K) TITRATIONS USING SERA FROM SENSITIZED INDIVIDUALS

Injected sera					P-K	Reactions following skin test with allergen (1 μg/ml) at serum dilutions:								
Donor	Group	Date (day/month/year)	Weeks	Total IgE (μg/ml)	Recipient	1:5, heated*	1:5,ads.†	1:5	1:10	1:20	1:40	1:80	1:160	>1:160
F.A.	A	11/12/69	30½	31	D.G.M.	0	N.D.	±	0	0	0	0	0	0
F.A.	A	19/8/70	66	87	D.G.M.	0	±	4+	3+	3+	2+	1+	±	0
J.McC.	B	19/3/71	96½	345	P.N.	0	0	4+	3+	2+	1+	±	0	0
D.M.	C	23/7/70	62½	56	P.S.	0	N.D.	3+	1+	±	0	0	0	0

N.D. = Not determined.

Controls, consisting of pre-treatment (0 week) sera and tris-albumin diluent, were always negative. See footnote to Table 2 for grading of skin reactions.

* Diluted serum incubated for 4 hours at 56° and pH 7·5.

† All detectable IgE was removed by adsorption on an anti-IgE immunosorbent. These samples produced slight non-specific erythema which persisted until the time of the allergen challenge. It was difficult to distinguish between a ± and a negative reaction, but all reactions were less than 1+.

with native allergen. Sera, taken at times when all three people were highly sensitive, showed high P-K titres of passively transferable reaginic antibody which was heat-labile and could be completely removed by immunoadsorption with anti-IgE. Fig. 6 compares the P-K titres of serum samples from subject F.A. with her leucocyte sensitivity during

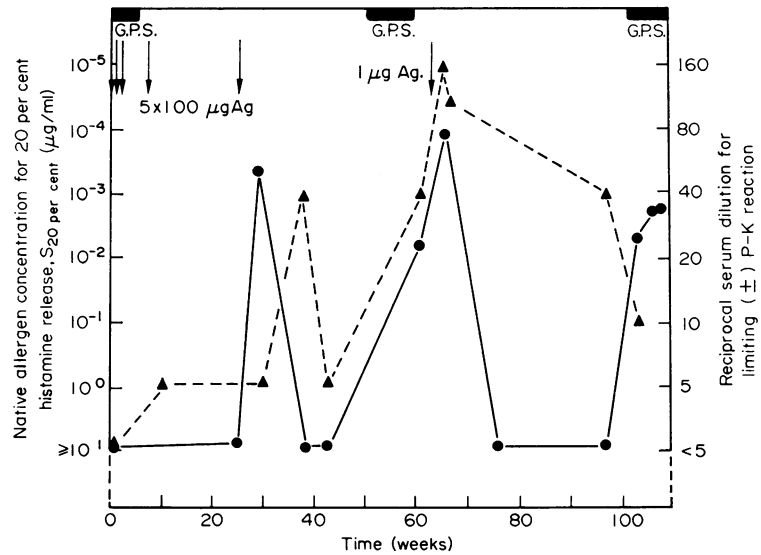

FIG. 6. Comparison of the time courses of leucocyte sensitivity and P-K titre toward native allergen for subject F.A. of Group A. Non-allergic recipient D.G.M. was tested in a single experiment with all sera. ●, Leucocyte sensitivity; ▲, P-K titre.

the course of the study. The leucocyte sensitivity of F.A. to allergen was high at 29½ weeks, coinciding with high skin sensitivity (Fig. 3), but the P-K titre of her serum at this time was only 1:5, for a minimal (±) skin reaction to injected allergen. Subsequently, F.A.'s P-K titre increased and thereafter showed a rough correlation with her leucocyte sensitivity.

DISCUSSION

The present study shows that clinically non-allergic individuals are capable of developing a reaginic antibody response, characterized by immediate skin and leucocyte sensitivities, following parenteral immunization with the major (Group I) grass pollen allergen or its allergoids. The particular modified allergen derivatives known as allergoids show greatly reduced allergenic reactivity in allergic people (Marsh, Lichtenstein and Campbell, 1970). The total quantities of antigen injected were similar to those used routinely in the immunotherapy of allergic individuals.

The induced reagin response was shown to be mediated by IgE antibody, since the skin sensitivity was passively transferable to the skin of non-allergics using sensitized individuals' sera (P-K test), and the P-K activity of the sera was destroyed either by heating at 56° for 4 hours or by adsorption with an anti-IgF solid phase immunosorbent. Despite the

development of pronounced skin reactivity and (in all but one of nine individuals immunized) leucocyte responsiveness characteristic of immediate hypersensitivity, there were no signs of clinical allergy in any of the people during natural exposure to grass pollen. Delayed skin reactions were also observed in most individuals.

Development of immediate skin and leucocyte reactivity in the non-allergic individuals was not observed until after the administration of much higher doses of antigen than encountered by natural exposure to pollen grains. In the present study, we usually needed 5×100 μg parenteral injections of antigen, of which 400 μg was administered with alumina gel adjuvant. Conversely, antigenic stimulation by natural means is transmucosal and occurs over several weeks in extremely low dosage in absence of adjuvants. In the Baltimore area, calculation of the total yearly adult dosage of grass Group I allergens suggests that it is below 1 μg (Marsh, 1971b).

Other workers have demonstrated immediate skin reactivity in non-allergic individuals following parenteral and/or transmucosal administration of relatively high doses of crude aqueous antigens such as ascaris extract (Brunner, 1934; Kailin, Davidson and Walzer, 1947) and heterologous serum (Cooke and Spain, 1929), and using purified antigens such as CB-1A from cotton seed (Spies and Bernton, 1962), diphtheria and tetanus toxoids (Kuhns, 1962), insulin (Tuft, 1928), keyhole limpet haemocyanin (Salvaggio, Castro-Murillo and Kundur, 1969) and dextran (Kabat, Turino, Tarrow and Maurer, 1957; Salvaggio, Kayman and Leskowitz, 1966). Also, oil-emulsified extracts of whole timothy grass and ragweed pollens induced immediate skin sensitivity in a good percentage of non-allergic humans (Sparks, Feinberg and Becker, 1962). As in the present study, several of these previous workers observed significant variations from person to person in the rate of development and the degree of skin sensitivity in artificially sensitized individuals. It is probable that the skin reactivity observed in these cases was also due to IgE antibody, especially since the reactivity of highly sensitized individuals was found to be passively transferable by serum injection into the skin of non-sensitized humans (Cooke and Spain, 1929; Spies and Bernton, 1962; Kuhns, 1962; Sparks *et al.*, 1962).

The induction of leucocyte responsiveness to injected antigen has not previously been observed in non-allergic humans. However, our observations confirm previous findings that non-atopic humans, who become highly skin sensitive following parenteral immunization with pollen extracts or castor bean allergen, do not develop clinical symptoms following inhalation of the immunizing antigens (Sparks *et al.*, 1962; Spies and Bernton, 1962). The complete explanation for the difference between naturally sensitized allergic individuals and our artificially sensitized 'non-allergics' with respect to allergic symptomatology is probably complex. However, a partial explanation may be that naturally and artificially sensitized people have quite different levels of anti-allergen IgG 'blocking' antibodies in their sera, despite many of the artificially sensitive people having levels of reaginic antibody-mediated skin sensitivity comparable to naturally sensitive people. We have already briefly reported (Marsh, 1971a) the existence of substantial levels of anti-Group I allergen 'blocking antibody' in the sera of most artificially sensitized individuals, whereas naturally grass-allergic individuals (even after extensive immunotherapy with aqueous crude grass pollen extracts) have very low or undetectable anti-Group I blocking antibody in their sera (unpublished data).

In the present studies, immunization was carried out with Group I allergen and allergoids adsorbed on alumina gel, a substance known to be a good adjuvant for induction of IgE-like reaginic antibody in several animal species, including rabbits (Revoltella and

Ovary, 1969; Strejan and Campbell, 1970), rats* and mice (Levine and Vaz, 1970). We have shown that such pollen antigens adsorbed on alumina also effectively induce IgE antibody production in non-allergic man, providing the antigenic stimulation is adequate. Other workers (Cooke, Loveless and Stull, 1937; Fitzgerald and Sherman, 1949) failed to induce detectable reaginic antibody in non-allergic man by injection of large doses (up to 10^6 PNU) of aqueous whole grass or ragweed pollen extracts. It therefore seems probable that alumina gel acts as an adjuvant for induction of IgE antibody response in man, although an appropriately controlled study will be required to confirm this point.

In naturally allergic individuals, there is a fairly good statistical correlation between skin and leucocyte sensitivities to pollen allergens, measured at the same time (Lichtenstein, Norman and Winkenwerder, 1971). In the artificially sensitized people, we failed to observe such a correlation when skin and leucocyte sensitivities were determined against any of the three immunizing antigens (Figs 2 and 3). Our results show that, in artificially sensitized people, both leucocyte responsiveness and the presence of substantial levels of IgE reaginic antibody in the serum (detectable by the P-K test) are *usually* short-lived in the absence of antigenic stimulation, in contrast to their persistence in naturally sensitized individuals† (Lichtenstein, King and Osler, 1966; Levy and Osler, 1967). Conversely, the observed skin reactivity of the artificially sensitized people decreased rather slowly over many weeks, with marked skin sensitivity still evident in most individuals at 35–40 weeks following the last immunization (Fig. 3). This finding agrees with the results of Brunner (1934) and Kailin, Rossbach and Walzer (1950) who noted that artificially induced skin reactivity often persisted for 6 months to 2 years after sensitization.

The above findings are consistent with artificially induced IgE antibody biosynthesis being relatively short-lived in contrast to the prolonged biosynthesis of naturally induced IgE antibody in allergic individuals. The half-life of IgE immunoglobulin in serum is known to be about 2-3 days (Waldmann, 1969), and one might hypothesize that the half-life of the IgE-fixing basophilic fraction of the leucocytes, considered to be responsible for allergen-mediated histamine release (Ishizaka, Tomioka and Ishizaka, 1970; DeBernardo, Lichtenstein and Ishizaka, 1971), is similarly short. (Brecher, von Foerster and Cronkite, 1962, found the half-life of circulating granulocytes to be 7 hours.) Thus, following a relatively short burst of IgE antibody biosynthesis after an immunization, leucocyte responsiveness and serum IgE antibody level should rise and decline in a similar fashion—a prediction only partially in accord with the observed results in one individual (Fig. 6). On the other hand, the persistence of skin reactions is probably due to both the high sensitivity of skin tests and the long lifetime of IgE-fixing mast cells in the skin. Passive sensitization is known to persist in the skin of recipients for many weeks, 1 per cent of the 2-day titre still being present at 10–15 weeks after injection (Cass and Andersen, 1968).

Our leucocyte assays show that individuals immunized with the native allergen became more sensitive to the immunizing antigen than those immunized with the allergoids and, furthermore, at all times the *allergoid*-immunized were more sensitive to *allergen* than the allergoid toward which they had been immunized (Fig. 5). These findings are consistent with the hypothesis that 'normal' individuals have been subliminally immunized by natural exposure to the allergen (i.e. IgE-producing cells have already been primed to allergen, but not to allergoid, and preferentially produce anti-allergen antibody when stimulated by

* Strejan and Cowley, personal communication.

† Occasionally we have noted that leucocyte responsiveness of a few naturally allergic individuals spontaneously 'turns off' without immunotherapy, although skin reactivity persists after the cells become unresponsive.

injection with the cross reacting allergoids)*. However, as one might expect, allergoid-immunized people have lower allergoid : allergen sensitivity ratios than allergen-immunized people (Fig. 5) and, following antigenic reinjection, these ratios tend to shift toward greater relative sensitivity to the immunizing antigen (cf. Fig. 4).

Our studies clearly show that the reaginic antibody response can be boosted by intracutaneous injection of antigen. Two equivocal cases where IgE antibody production may have been stimulated merely by natural exposure to grass pollen grains warrant further investigation, since they suggest that the nasal mucosa of non-allergic individuals are sufficiently permeable to allow for the possibility of natural allergenic stimulation of IgE-antibody production.

In conclusion, we have confirmed that clinically non-allergic individuals are able to develop IgE-mediated hypersensitivity reactions, but not clinical symptoms, following parenteral administration of antigen. However, the dosage of the major Group I rye grass pollen allergen (when combined with alumina gel adjuvant) which is required to achieve this result is very substantially ($\geqslant$500-fold) greater than the annual dosage (under 1 μg) of Group I grass pollen allergens toward which naturally allergic individuals become sensitized (cf. Marsh, 1971b).

ACKNOWLEDGMENTS

We are indebted to Dr Kimishige Ishizaka for performing the immunoadsorptions of IgE and determination of IgE levels. We particularly thank Joanne Tignall and also Paul Black, Laverne Grove, Jeanne Harrison, Marcia Lyons and Anne Sobotka for technical help and all the volunteers who continued with the study for over 2 years.

This research was supported by USPHS Grants Nos AI09565, AI08270, AI07290 and AI10304 from the NIAID and Research Career Development Awards AI50304 and AI42373.

Parts of this study were presented at the American Academy of Allergy Meeting, 18th February, 1970, at the 8th Symposium of the Collegium Internationale Allergologicum, 16th May, 1970, and at the Federation Meetings, 14th April, 1971.

Part of this study was performed in the O'Neill Memorial Laboratories of The Good Samaritan Hospital (Contribution No. 13).

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* A further explanation for these interesting findings is that, since the allergoids consist of a heterogeneous mixture of antigens and the allergen is homogeneous (Marsh, 1971a), the molecules within the allergoid sub-populations would be more efficient in collectively stimulating cross-reactive anti-allergen antibody than antibody against each molecule within the allergoid sub-populations.

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