

Immunologic response of atopic and normal individuals to keyhole limpet hemocyanin

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Groups of atopic and normal individuals were immunized intranasally with aerosolized keyhole limpet hemocyanin (KLH), a purified high-molecular weight protein known to be highly immunogenic in man. A high percentage of atopic, as opposed to normal, individuals demonstrated immediate wheal and flare skin reactivity to KLH during the process of patient selection prior to initiation of the study. We divided 65 subjects with negative KLH wheal and flare skin tests into "normal," "atopic," and "family history" groups. KLH was administered: (1) by weekly nasal aerosol (0.3 mg. per week for 2 months followed by a 0.6 mg. aerosol "booster"); (2) by monthly intracutaneous injection of 22 µg. At 2 months, wheal and flare skin reactivity appeared with greater frequency in atopic individuals irrespective of immunization route. By 2½ months, group differences were no longer evident. Many subjects developed large delayed skin reactions to KLH after one month, and these were usually followed by the appearance of immediate reactions at the 2 month testing. Low level mercaptoethanol (ME) - sensitive (IgM) anti-KLH hemagglutinating antibody coincided with appearance of delayed skin reactions early in the course of the study and was followed sequentially by wheal and flare skin reactivity and IgG hemagglutinating antibody. IgG anti-KLH titers and wheal and erythema skin reactivity were greatest at the termination of the study.

In previous studies, subcutaneous immunization of atopic and normal individuals with purified ribonuclease and dextran resulted in similar group immunologic responses.¹⁻⁴ However, long-term intranasal immunization with these antigens in aerosolized form resulted in the appearance of wheal and flare skin reactivity predominantly among atopic subjects.

These results suggested that atopic and normal individuals differed in

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their manner of handling antigen coming in contact with mucosal tissue.

In the current project, these observations were extended to intranasal and intracutaneous immunization with the high-molecular weight protein keyhole limpet hemocyanin (KLH).

Absorption of unaltered antigen across mucosal barriers has been reported to vary with molecular size,⁵ and the current project was designed to compare results of intranasally administered KLH with previous results when antigens closely approximating those of "natural" pollen antigens in size were used.

MATERIALS AND METHODS

Group selection

Five groups of volunteer subjects (criteria for group selection were previously described⁴) were used as follows: Group I consisted of 14 patients with allergic rhinitis. Group II consisted of normal medical student volunteers with no present, past, or family history of atopic disease. Group III consisted of 10 individuals with no present or past history of atopic disease but one or more immediate family members (parents or siblings) with bronchial asthma or allergic rhinitis. Group IV consisted of 13 subjects fulfilling the criteria of Group I. Group V consisted of 11 subjects fulfilling the criteria of Group II.

Each subject was tested by scratch and/or intradermal tests (1:10 w/v and 1:200 w/v extracts, respectively) with a battery of 14 common local inhalant allergens and a saline control. Only individuals with a broad pattern of positive wheal and flare reactions plus a personal history of rhinitis were placed in the atopic groups (I and IV). Potential "normal" volunteers with one or more positive wheal and flare skin reactions were excluded from the normal and family history groups.

Antigen

KLH* (*Megathura crenulata*) was employed. Associated KLH was prepared by repeated centrifugation and dialysis as previously described.⁶ No preservative was added since phenol and other additives were found to discolor and denature the antigen in solution. Stock antigen (20 mg. per milliliter) was stored under sterile conditions at 4° C.

Immunization procedure

Groups I, II, and III were given 4 intranasal inhalations of KLH per nostril, weekly for a 2 month period. A No. 40 DeVilbiss nebulizer was used, and approximately 300 μ g were given per week over the 2 month period followed by a 600 μ g aerosol "booster" at 2½ months. The subject's head was tilted backward at a 45 degree angle during aerosolization to facilitate retention of the solution.

Groups IV and V were given only monthly intracutaneous injections of 22 μ g of KLH in the form of skin tests to assess the immunologic response to repeated intradermal tests per se.

*Obtained from Pacific Biomarine Company, Venice, Calif.

Intradermal tests

Methods of testing and recording reactions were as previously described.¹⁻⁴ Two concentrations of KLH were injected per test, 20 μ g and 2.0 μ g in 0.02 ml. volumes. Tests were performed prior to immunization and at 1, 2, and 2½ months. Reactions were read after 20 minutes, 24 hours, and 48 hours. They were recorded in millimeters of erythema (er.) and edema (ed.) (immediate) and erythema and induration (delayed).

Hemmagglutinating-antibody analysis

Bleedings were done prior to each skin test. After clotting overnight and centrifugation, sera were frozen with no added preservative and absorbed with fresh normal rabbit cells.

Tests for hemagglutinating antibody were performed with formalinized erythrocytes with the use of the microhemagglutination method of Takátsy.⁷ Rabbit erythrocytes collected in Alsever's solution and formalinized according to the method of Csizmas⁸ were employed. KLH in a concentration of 1.5 mg. per milliliter (found to be optimum for cell coating with the microtechnique and formalinized cells) was coupled to the formalinized erythrocytes with bis-diazotized benzidine. Sera were decomplexed and absorbed for 20 minutes at room temperature with 50 per cent rabbit cells in phosphate buffer prior to use.

IgM- and IgG-hemagglutinating activity was distinguished on the basis of sensitivity to 0.133M 2-ME with recognition that such a distinction was not absolute and that some IgA and IgE might also be affected by ME treatment.^{9,10} Specific neutralization with anti-heavy chain immunoglobulin sera was not done, but Levine and co-workers,¹¹ with the use of a similar but more sensitive method to detect benzylpenicilloyl (BPO)-specific antibody in man, have shown by neutralization techniques that ME-sensitive antisera contained virtually only IgM antibody. Serum dilutions were mixed directly with 0.133M 2-ME in buffer and incubated overnight at 4° C. No dialysis or sulfhydryl inactivation was necessary since this ME concentration did not interfere with titers below 1:8.

Skin sensitizing-antibody

Antibody was measured by the Prausnitz-Küstner (P-K) method. Sera were frozen at -20° C., thawed, and centrifuged at 2,000 r.p.m. for one hour prior to use. Approximately 0.05 ml. of serum diluted 1:10, 1:25, 1:50, 1:100, 1:200, and 1:600 in saline were injected into the skin of nonatopic volunteers. Four P-K assays were performed per volunteer. Sites were challenged at 24 hours with 0.02 ml. of KLH (approximately 2.0 μ g of KLH per test). Reactions were read at 20 minutes. Saline controls were done in all cases.

RESULTS**Skin reactivity**

Although KLH has been shown to elicit a primary response in other studies,¹² many of our volunteer subjects exhibited wheal and flare or delayed

skin reactions to KLH on the preimmunization testing. Of 50 such volunteers who were tested in the process of selection for the study, 51 per cent of those with atopy and 13 per cent of normal subjects demonstrated positive wheal and flare reactions to KLH, a statistically significant group difference ($p = 0.02$; $\chi^2 = 6.35$). These individuals were not selected for the study. Ten of these KLH-positive subjects were bled and all demonstrated hemagglutinating-anti-KLH activity primarily of the ME-resistant type.

Development of immediate skin reactivity over a 2½ month period among subjects selected for the study is illustrated in Tables I and II. In the atopic

Table I. Wheal and flare skin reactions to KLH during the course of intranasal immunization

Group and patient No.*	Skin reactivity (mm.)			
	Preimmunization	1 Month	2 Months	2½ Months
<i>Group I</i>				
A1	slight er.	0	0	—†
A2	0	0	10 ed. 45 er.	12 ed. 45 er.
A3	0	0	—	—
A4	0	0	0	9 ed. 18 er.
A5	0	0	slight er.	6 ed. 31 er.
A6	0	slight er.	10 ed. 10 er.	10 ed. 29 er.
A7	0	0	6 ed. 12 er.	10 ed. 36 er.
A8	0	14 er.	16 ed. 25 er.	14 ed. 47 er.
A9	0	0	10 ed. 19 er.	8 ed. 19 er.
A10	0	9 ed. 13 er.	9 ed. 14 er.	10 ed. 33 er.
A11	0	0	9 ed. 9 er.	0
A12	0	0	7 ed. 16 er.	13 ed. 49 er.
A13	0	0	0	10 ed. 36 er.
A14	0	0	0	0
<i>Group II</i>				
N1	0	0	0	—
N2	0	0	0	10 ed. 10 er.
N3	0	0	0	7 ed. 7 er.
N4	0	0	0	0
N5	0	0	0	—
N6	0	0	0	0
N7	0	0	0	6 ed. 41 er.
N8	0	0	6 ed. 6 er.	9 ed. 9 er.
N9	0	0	8 ed. 23 er.	10 ed. 22 er.
N10	0	0	0	0
N11	0	0	10 ed. 23 er.	0
N12	0	0	0	9 ed. 9 er.
N13	0	0	—	—
N14	0	0	—	—
<i>Group III</i>				
F1	0	0	0	6 ed. 10 er.
F2	0	0	12 ed. 28 er.	10 ed. 36 er.
F3	0	0	0	—
F4	0	0	8 ed. 32 er.	12 ed. 50 er.
F5	0	0	6 ed. 39 er.	10 ed. 30 er.
F6	0	0	0	7 ed. 12 er.
F7	0	0	0	9 ed. 27 er.
F8	0	0	0	11 ed. 34 er.
F9	0	0	15 ed. 15 er.	13 ed. 35 er.
F10	0	0	0	0

*A = atopic; N = normal; F = family history.

†Dash indicates test was not done.

Table II. Wheal and flare skin reactions to KLH during the course of intradermal* immunization

Group and patient No.†	Skin reactivity (mm.)			
	Preimmunization	1 Month	2 Months	2½ Months
<i>Group IV</i>				
A1	0	0	0	10 ed. 25 er.
A2	0	0	0	9 ed. 11 er.
A3	0	0	0	10 ed. 22 er.
A4	0	7 ed. 24 er.	10 ed. 25 er.	5 ed. 19 er.
A5	0	10 ed. 32 er.	10 ed. 25 er.	8 ed. 11 er.
A6	slight er.	slight er.	7 ed. 22 er.	9 ed. 35 er.
A7	0	0	0	—
A8	0	0	8 ed. 20 er.	0
A9	0	0	0	9 ed. 9 er.
A10	0	0	0	22 ed. 22 er.
A11	0	8 ed. 14 er.	7 ed. 8 er.	—
A12	0	0	6 ed. 16 er.	8 ed. 30 er.
A13	0	0	7 ed. 25 er.	8 ed. 20 er.
<i>Group V</i>				
N1	0	0	0	slight er.
N2	0	5 ed. 11 er.	10 ed. 19 er.	20 ed. 32 er.
N3	0	0	10 ed. 21 er.	10 ed. 31 er.
N4	0	0	—	—
N5	0	0	0	6 ed. 25 er.
N6	0	0	0	0
N7	0	0	8 ed. 12 er.	9 ed. 25 er.
N8	0	0	0	0
N9	0	0	0	10 ed. 18 er.
N10	0	0	0	0
N11	0	0	0	—

*KLH, 22.0 µg, was given monthly in the form of skin tests.

†A = atopic; N = normal.

groups, 8 of the 13 in Group I (aerosolized) and 7 of 13 in Group IV (intradermal) developed wheal and flare immediate skin reactivity to KLH after 2 months. Among the "normal" subjects, 3 of 12 in Group II (aerosolized) and 3 of 10 in Group V (intradermal) developed positive wheal and flare reactions at this time. The number of subjects per group was small for adequate statistical evaluation, and differences between the atopic and normal aerosolized groups (I and II) and intradermally immunized groups (IV and V) were of borderline statistical significance ($p = 0.1$; $\chi^2 = 3.38$ for Groups I and II, and $p = 0.25$; $\chi^2 = 1.23$ for Groups IV and V, respectively). However, when atopic versus normal subjects are considered without regard for immunization route, 15 of 26 atopic as opposed to 6 of 22 normal individuals developed wheal and flare skin reactivity by 2 months, a significant difference ($p = 0.05$; $\chi^2 = 4.48$). In addition, the elimination of many KLH-positive atopic individuals from the study during the process of selection likely weighted our results against the demonstration of group differences.

After 2 months, 4 out of 10 (40 per cent) subjects in Group III (family history) developed positive wheal and flare skin reactions to KLH, a response intermediate between that noted for the atopic aerosolized group (I) and normal aerosolized group (II).

At the 2½ month testing, a majority of individuals among all test groups manifested positive wheal and flare skin reactions to KLH as follows: Group I = 10/12 (84 per cent); Group II = 6/10 (60 per cent); Group III = 8/9 (88 per cent); Group IV = 10/11 (90 per cent); and Group V = 9/9 (66 per cent).

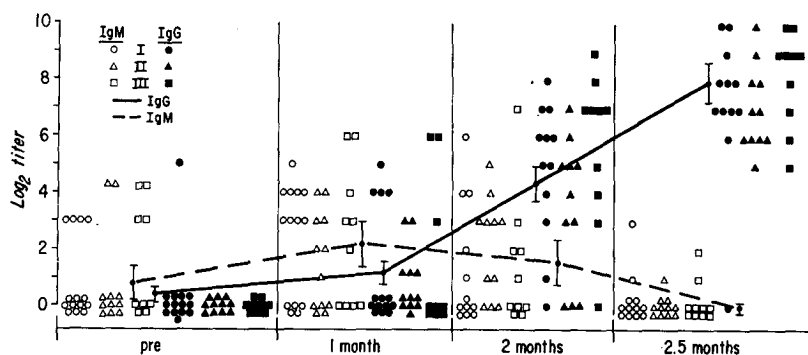
Large delayed skin reactions (up to 60 mm. induration and erythema) appeared in most members of all groups during the course of the study. These reactions were detected at the one-month testing in most cases. They were maximal at 24 hours, receded rapidly despite their large size, but resulted in a residual bluish discoloration at the test site for several days in many subjects. Skin test-punch biopsies taken at 24 hours in 2 subjects with large delayed reactions revealed prominent mononuclear cell infiltrates in a definite perivascular distribution compatible with typical delayed reactions. These reactions generally preceded the development of wheal and flare skin reactivity by one month during the course of immunization, which suggested that they were of the transient or Jones-Mote variety.^{13, 14} Of those individuals among all groups who manifested large delayed reactions to KLH, 90 per cent developed immediate wheal and erythema reactions usually subsequent to, but in some cases together with, the appearance of delayed reactions. In 38 per cent, delayed reactions were no longer noted after the appearance of wheal and erythema reactions.

Symptoms

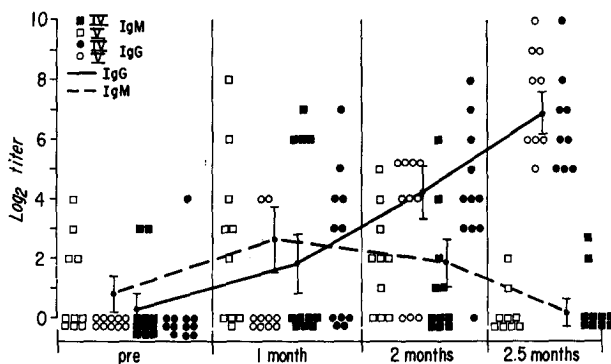
Medical student No. 3 of Group I discontinued the study because of rhinorrhea, malaise, and development of "tender submental and cervical nodes" after the ninth aerosol. Subject No. 8 of Group I developed profuse rhinorrhea of 24 hours' duration after the eighth aerosol. It is of some interest that this individual demonstrated intense wheal and flare skin reactivity early in the course of the study, developed the largest wheal and flare skin reactions of all patients in the study, and was the only subject whose serum demonstrated positive P-K activity in a significant titer. Subject No. 7 of Group I developed rhinorrhea for several hours after the ninth aerosol, and subject No. 4 of Group III, who developed a large wheal and flare skin reaction at the 2½ month testing, complained of pruritus and pain of 42 hours' duration involving the entire upper extremity. No other subject reported upper respiratory symptoms after aerosol exposure or excessive local inflammatory signs after intradermal testing during the course of the study.

Hemagglutinating antibody

Results of ME-resistant and sensitive anti-KLH activity in all groups are illustrated in Figs. 1 and 2. Group responses were similar throughout the course of the study. Low ME-sensitive anti-KLH titers were noted in some subjects prior to immunization and were first detected in others at the one-month bleeding. After 2 months, ME-resistant anti-KLH activity was noted in most patients. ME-sensitive activity persisted in some patients at this time and was detected initially in others who had exhibited no previous hemagglutinating activity or wheal and flare skin reactivity. At 2½ months, ME-resistant

**Fig. 1**

IgG and IgM anti-KLH hemagglutinating activity in groups immunized by intranasal aerosol. Trend lines represent combined group IgG and IgM mean values with standard deviation.

**Fig. 2**

IgG and IgM anti-KLH hemagglutinating activity in groups immunized by intradermal injection. Trend lines represent combined group mean IgG and IgM values with standard deviation.

activity was maximal in all subjects, and the ME-sensitive response had ceased.

In general, low ME-sensitive titers coincided with the appearance of delayed skin reactions early in the course of the study and were followed sequentially by wheal and flare skin reactivity and ME-resistant anti-KLH activity. ME-resistant anti-KLH titers and wheal and erythema skin reactivity were highest at the termination of the study (2½ months).

Passive transfer activity

Subject No. 8, Group I, demonstrated a P-K titer of 1:250 at 2½ months. It is noteworthy that this individual demonstrated the largest wheal and flare skin reactions of all subjects and was the only member of all groups who developed severe nasal symptoms during the course of the study.

Two atopic subjects with large preimmunization skin reactions to KLH (12 ed., 43 er., and 12 ed., 34 er., at the 0.2 µg test dose) also manifested positive

P-K activity in low titers of 1:5 and 1:50, respectively. No other subjects demonstrated positive P-K activity at the 2 or 2½ month bleeding.

DISCUSSION

Our results are consistent with previous reports^{1, 3, 15} which demonstrate that normal as well as atopic individuals can develop wheal and flare skin reactivity after prolonged exposure to small quantities of intranasally aerosolized antigens. Assuming that most ME-sensitive hemagglutinating activity in our study represented IgM anti-KLH antibody and that our observed delayed skin reactions represented transient or Jones-Mote reactions, our results also suggest that the immune response of normal and atopic individuals to KLH administered in repeated small doses intradermally, or via nasal aerosol, progresses through the normal sequence of an early IgM and delayed hypersensitivity response followed by an IgG response, as has been shown with the subcutaneous immunization route.^{12, 16}

Wheal and flare skin reactivity appears to be coincidentally within the framework of the immune response to KLH and generally follows the development of delayed hypersensitivity and ME-sensitive hemagglutinating activity. Its appearance does not seem to be influenced by the mode of immunization with KLH, since a similar incidence of wheal and flare skin reactivity develops in groups of atopic subjects after repeated intradermal injection or intranasal inhalation.

The brisk immunologic response of our atopic individuals to microgram quantities of KLH administered intradermally in the form of monthly skin tests is in keeping with its high immunogenicity in man and precludes any assessment of mucosal permeability to intranasally aerosolized KLH. Thus, it may be that KLH is not an appropriate antigen to provide the most definitive results in this type of study. This difficulty serves to emphasize that the only fruitful approach to a problem of this type is to immunize similar groups of atopic and normal subjects with antigens of many types and molecular weights with the use of a wide range of concentrations and several routes of immunization.

Hansen and Gleich¹⁷ have shown that the immunologic response of atopic and normal individuals to intranasally aerosolized hemocyanin from *Limulus polyphemus* is similar. However, different immunization schedules, sources of antigen, population locations, and antigen concentrations preclude comparison of their results with ours. The significant increase in "preimmunization" wheal and flare skin reactivity to KLH noted among atopic subjects when compared with normal individuals of the Gulf South area is noteworthy, since these individuals were not likely exposed to *Megathura crenulata*, an inhabitant of southern California Pacific coastal waters, and immunologic cross-reactivity with other Atlantic mollusk hemocyanins has not been reported to occur.¹⁸ Residents of southern Louisiana regularly consume large quantities of such hemocyanin-containing invertebrates as crayfish, oyster, crab, and shrimp, and it is likely that considerable cross-reactivity exists. Assuming there is cross-reactivity, this finding suggests greater absorption of hemocyanins via the

gastrointestinal tract in atopic individuals as a result of "natural" exposure to seafood products. These results are also in agreement with the observations of Barrick and Farr,¹⁹ who reported a significantly higher incidence of antimilk protein antibodies among atopic as opposed to normal individuals. Although this hypothesis affords a simple explanation of our findings, one might reasonably assume that a generalized mucosal permeability defect would be operative for proteins with molecular weights in the range of common natural airborne allergens and food antigens rather than high-molecular weight KLH, which would not likely penetrate mucosal barriers. Indeed, administration of bovine ribonuclease (a protein antigen with a molecular weight approximating that of several "natural" airborne allergens) via intranasal aerosol readily induces wheal and flare skin reactivity in atopic as opposed to normal subjects.^{2, 15}

The high incidence of preimmunization wheal and flare activity among our atopic subjects might also be explained by postulating an altered reactivity to pharmacologic intermediates released by antigen-antibody reactions particularly in view of the relatively small size of these wheal and flare skin reactions. However, the presence of ME-resistant hemagglutinating activity in the sera of these subjects and the positive P-K reactions in 2 subjects with large preimmunization wheal and flare reactions mitigate against this hypothesis. In addition, previous work with histamine and the histamine releaser 48/80 revealed no difference in wheal and flare skin reactivity between groups of normal and atopic subjects.¹

Our findings might also reflect a simple quantitative difference between atopic and normal individuals which is brought to light by repeated administration of small quantities of antigen either intradermally or via mucosal application. Atopic persons would thus develop a heightened immune response to airborne allergens coming in contact with mucosal tissue in small quantities for a sufficient length of time. Under experimental conditions, this quantitative difference could be detected by repeated administration of small quantities of purified antigens via nasal aerosol or repeated intradermal inoculation. Direct skin tests are more sensitive indicators of skin-sensitizing antibody than passive transfer tests,^{20, 21} and the small number of reaginic antibody molecules necessary for skin sensitization would make this type of immunologic response more apparent. The recent demonstration of elevated levels of circulating IgE (IgND), the immunoglobulin currently thought to be associated with skin-sensitizing antibody, in atopic individuals is also in keeping with such a hypothesis.²²⁻²⁵

Finally, differences in antibody production to KLH with respect to immunoglobulin class might account for some of our observed group differences, as illustrated in Fig. 3. Although the general pattern of ME-sensitive and ME-resistant anti-KLH hemagglutinating activity was similar in atopic and normal individuals, it can be seen that atopic individuals (regardless of immunization route) developed ME-resistant hemagglutinating titers (assumed to represent primarily IgG activity) in proportion to intensity of IgE mediated wheal and flare skin reactivity as opposed to normal individuals who demonstrated comparable ME-resistant hemagglutinating titers but less wheal and flare skin

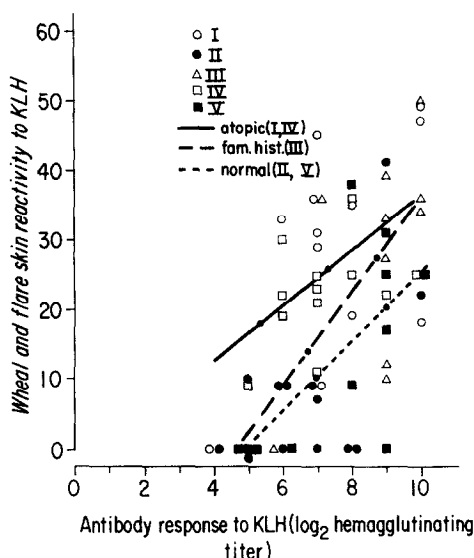


Fig. 3

Relationship between hemagglutination anti-KLH titer and wheal and flare skin reactivity. Values represent greatest degree of erythema and highest hemagglutination titer noted in individual subjects during course of study (2½ months in almost all cases). Trend lines indicate relationship between immediate skin reactivity and hemagglutination titer for Groups I and IV (atopic), Group III (family history), and Groups II and V (normal). At the adjusted mean log₂ hemagglutinating titer for all groups of 7.5, mean diameter of wheal and flare skin reactivity was 26.5 mm. (atopic groups), 21.6 mm. (family history group), and 12.6 mm. (normal groups). Differences between atopic and normal groups were significant at the one per cent level ($p < 0.01$).

reactivity. Such "qualitative" differences in antibody production with respect to immunoglobulin class are thought to influence the type and extent of immediate allergic reactions in penicillin allergy. Levine and co-workers^{26, 27} have shown that IgG hemagglutinating antibodies (ME-resistant) have a protective effect against development of IgE-mediated benzylpenicilloyl (BPO)-specific wheal and flare skin reactions. When these patients are given increasing doses of penicillin in an attempt at "desensitization," they develop symptoms only after several hours coincident with a fall in the titers of IgG antibody. High "protective" ME-resistant IgG log₂ titers demonstrable at the 2½ month bleeding in our subjects with wheal and flare skin reactivity to KLH could explain the absence of systemic immediate allergic reactions and paucity of nasal symptoms upon repeated intranasal exposure, as in the case of BPO-allergic subjects. A more plausible explanation for the absence of nasal symptoms in our KLH-sensitive subjects after repeated intranasal exposure to this antigen would be to assume that some circulating hemagglutinating activity represented "protective" IgA anti-KLH antibody which gained access to nasal mucous membranes as secretory IgA (or was synthesized locally together with transport piece of Tomasi and associates²⁸). Attempts to correlate secretory IgA anti-KLH activity with allergic symptoms in such subjects should be of interest and are in progress.

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REFERENCES

1. Leskowitz, S., and Lowell, F. C.: A comparison of the immunologic and physiologic responses of normal and allergic individuals, *J. ALLERGY* 32: 152, 1961.
2. Salvaggio, J. E., Cavanaugh, J. J. A., Lowell, F. C., and Leskowitz, S.: A comparison of the immunologic responses of normal and atopic individuals to intranasally administered antigen, *J. ALLERGY* 35: 62, 1964.
3. Salvaggio, J., Kayman, H., and Leskowitz, S.: Immunologic responses of atopic and normal individuals to aerosolized dextran, *J. ALLERGY* 38: 31, 1966.
4. Salvaggio, J. E., and Leskowitz, S.: A comparison of the immunologic responses of normal and atopic individuals to parenterally injected, alum precipitated protein antigen, *Internat. Arch. Allergy* 26: 264, 1965.
5. Bernstein, I. D., and Ovary, Z.: Absorption of antigens from the gastrointestinal tract, *Internat. Arch. Allergy* 33: 521, 1968.
6. Campbell, D., Garvey, J., Cremer, N., and Sussdorf, D.: *Methods in immunology, a laboratory text for instruction and research*, New York, 1963, W. A. Benjamin, Inc., p. 69.
7. Takátsy, G.: The use of spiral loops in serological and virological micro-methods, *Acta microbiol. acad. sc.* 3: 191, 1955.
8. Csizmas, L.: Preparation of formalinized erythrocytes, *Proc. Soc. Exper. Biol. & Med.* 103: 157, 1960.
9. Leddy, J. P., Freeman, G. L., Asuncion, L., and Todd, R.: Inactivation of the skin-sensitizing antibodies of human allergy by thiols, *Proc. Soc. Exper. Biol. & Med.* 3: 7, 1962.
10. Rocky, J. H., and Kunkel, H. G.: Unusual sedimentation and sulfhydryl sensitivity of certain isohemagglutinins and skin-sensitizing antibody, *Proc. Soc. Exper. Biol. & Med.* 110: 101, 1962.
11. Levine, B., Fellner, M., Levytska, V., Franklin, E., and Alisberg, N.: Benzylpenicilloyl-specific serum antibodies to penicillin in man. II, *J. Immunol.* 96: 719, 1966.
12. Swanson, M., and Schwartz, R.: Immunosuppressive therapy: The relation between clinical response and immunologic competence, *New England J. Med.* 277: 163, 1967.
13. Dienes, L., and Schoeneit, E.: The reproduction of tuberculin hypersensitiveness in guinea pigs with various protein substances, *Am. Rev. Tuberc.* 20: 92, 1929.
14. Martins, A., and Raffel, S.: Cellular activities of hypersensitive reactions. II. Plasmopoiesis in delayed and "Jones-Mote" reaction sites, *J. Immunol.* 93: 948, 1964.
15. Schwartz, H. J., Leskowitz, S., and Lowell, F. C.: Studies on "intrinsic" allergic respiratory disease, with a hypothesis concerning its pathogenesis, *J. ALLERGY* 42: 169, 1968.
16. Bandilla, K., and McDuffie, F.: Immunoglobulin classes of antibodies produced in the primary and secondary responses in man, *Fed. Proc.* 27: 563, 1968.
17. Hansen, R. L., and Gleich, G. J.: A comparison of antibody formation in atopic and normal individuals, *J. ALLERGY* 39: 122, 1967. (Abst.)
18. Malley, A., Saha, A., and Halliday, W.: Immunochemical studies of hemocyanin from giant keyhole limpet (*Megathura crenulata*) and the horseshoe crab (*Limulus polyhemus*), *J. Immunol.* 95: 141, 1965.
19. Barrick, R. H., and Farr, R. S.: The increased incidence of circulating anti-beef albumin in the sera of allergic persons and some comments regarding the possible significance of this occurrence, *J. ALLERGY* 36: 374, 1965.
20. Redmond, A. P., and Levine, B. B.: The relationship between direct immediate skin tests and passive transfer tests in man. Studies in the Penicillin Allergy system, *J. ALLERGY* 39: 51, 1967.
21. Levine, B. B., and Fellner, M. J.: The nature of immune complexes initiating allergic wheal-and-flare reactions, *J. ALLERGY* 36: 342, 1965.
22. Ishizaka, K., and Ishizaka, T.: Physicochemical properties of reaginic antibody. I. Association of reaginic activity with an immunoglobulin other than γ A- or γ G-Globulin, *J. ALLERGY* 37: 169, 1966.
23. Ishizaka, K., Ishizaka, T., and Hornbrook, M.: Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity with γ E-globulin antibody, *J. Immunol.* 97: 840, 1966.
24. Johansson, S. G.: Raised levels of a new immunoglobulin class (IgND) in asthma, *Lancet* 2: 951, 1967.
25. Immunoglobulin E. A new class of human immunoglobulin, World Health Organization International Reference Centre for Immunoglobulins, Lausanne, Feb., 1968, noted in: *J. Immunol.* 100: 1143, 1968.
26. Levine, B.: Immunologic mechanisms of penicillin allergy. A haptenic model system for the study of allergic diseases of man, *New England J. Med.* 275: 1115, 1966.
27. Voss, H. E., Redmond, A. P., and Levine, B. B.: Clinical detection of the potential allergic reactor to penicillin by immunological tests, *J. ALLERGY* 37: 99, 1966. (Abst.)
28. Tomasi, T., Tan, E., Solomon, A., and Prendergast, R.: Characteristics of an immune system common to certain external secretions, *J. Exper. Med.* 121: 101, 1965.