Int. Arch. Allergy 47: 155–160 (1974)

Immune Response to Keyhole-Limpet Hemocyanin in the Human[1]

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Abstract. Delayed hypersensitivity was studied in twelve normal adults given a single intradermal dose of 100 μg keyhole-limpet hemocyanin (KLH). Minimal local reactions were observed, but no systemic reactions occurred. Eleven persons had (1) positive skin test, and/or (2) a small percentage of KLH-sensitized lymphocytes in the *in vitro* lymphocyte blastogenesis test 2 weeks after immunization and (3) hemagglutinins demonstrable by a passive hemagglutination test.

Introduction

Clinical evaluation of cellular immune response is still cumbersome and difficult, mainly because of problems associated with *in vivo* testing of patients and technical difficulties of *in vitro* methods for measuring such reactions. An expert committee of the World Health Organization reviewed this field recently and recommended the specific antigens 2,4-dinitrochlorobenzene (DNCB) and keyhole-limpet hemocyanin (KLH) for testing the ability to produce a delayed type hypersensitivity reaction. DNCB has been widely used in clinical tests, but we found its application irritant for the patient and the results difficult to evaluate. In contrast to DNCB, KLH has not been often used in humans, so that a critical evaluation concerning reliability, risk to the patient, and importance of skill and experience of the immunologist doing the test is needed.

[1] This work was supported by a grant from the Swiss National Fund (NF 3.559.71).

Received: October 29, 1973.

Materials and Methods

The subjects were twelve healthy adults aged 20–50 years with no previous history of allergic reaction after ingestion of shellfish or other seafood. Two had a history of atopic rhinitis which was inactive during the investigation. In addition, the serum and lymphocytes of one patient were used as a positive control. He had been hyperimmunized with 65 mg KLH coupled to purified growth hormone in 13 weekly doses given in an attempt to influence severe acromegaly due to an intractable eosinophilic tumor [GITZELMANN and STEINMANN, personal commun.]. His lymphocytes were cultured and stimulated with KLH to determine the optimal conditions (antigen dose, culture time, etc.) to be observed in testing the lymphocytes of the twelve normal subjects under investigation.

Antigen. KLH *(Megathura crenulata)* is prepared by repeated centrifugation and dialysis of the animal's hemolymph according to the method described by CAMPBELL *et al.* [1963] and can be obtained as an ammonium sulfate precipitate (Pacific Biomarine Supply Co., Venice, Calif.). This original material was prepared for use as antigen by dialysis against 0.15 M sodium chloride at pH 8.0 (0.01 M Tris buffer) at 0 °C and subsequently centrifuged at 30,000 *g* for 30 min to remove denatured protein. The protein nitrogen concentration in the supernatant was determined by the LOWRY method. The stock solution contained 24 mg protein/ml. Solutions containing 10 and 100 μg/ml were prepared by dilution with 0.15 M sodium chloride. They were sterilized by Millipore filtration (pore size 0.2 μm) and addition of merthiolate (1:10,000) and stored at 4 °C in 5-ml rubber-capped vials.

Immunization and skin tests. The twelve volunteers were tested with 1 μg KLH intradermally and after 20 min with 10 μg before immunization. None showed any immediate reaction. They were then immunized by the intradermal injection of 100 μg KLH into the volar surface of the forearm (5 wheals of 0.2 ml each). Delayed hypersensitivity was tested 7 and 14 days later by intradermal injection of 1 and 10 μg of the same material in the opposite forearm. Erythema and/or induration were measured 4, 24, and 48 h after injection, according to recommendations of the World Health Organization.

In vitro *lymphocyte blastogenesis.* The lymphocytes of each subject were stimulated *in vitro* with KLH and phytohemagglutinin (PHA) before and 7 and 14 days after immunization. DNA synthesis was measured as previously described by BODMER and HITZIG [1971]: 1.5×10^6 lymphocytes per culture in 1 ml BME and Hepes medium (in triplicate); incubation, 90 h; labelling time after addition of 2 μCi H^3-thymidine, 6 h. Antigen concentrations of 10 and 100 μg KLH or of 1 mg PHA per culture were used. Results with the specific antigen (KLH) were expressed as the percentage of incorporation after PHA stimulation.

Serum antibodies against KLH. Tests for precipitating antibodies were performed in double-diffusion Ouchterlony plates, using an antigen concentration range of 0.01–24 mg/ml and serum dilutions of 1:1 to 1:64. The passive hemagglutination test was done with tanned sheep erythrocytes coated with KLH (optimum concentration for coating 0.06 mg KLH/ml) according to the method described by STAVITSKY and ARQUILLA [1958]. The tests were done in micro U-plates (Cooke & Co.) and read after incubation at room temperature overnight.

Table I. Delayed hypersensitivity reaction against KLH[1]

Subjects	Days after immunization				Result
	7		14		
	1 μg	10 μg	1 μg	10 μg	
1	0	6/6	0	8/9	+
2	0	10/10	0	10/10	+
3	0	0	0	0	−
4	0	10/10	0	ND	+
5	0	10/10	0	10/10	+
6	0	11/11	0	ND	+
7	0	15/15	ND	ND	+
8	ND	ND	2/2	15/15	+
9	5/5	ND	10/10	12/12	+
10	0	5/5	ND	12/12	+
11	8/8	ND	ND	12/12	+
12	0	24/24	ND	ND	+

[1] Immunization: 100 μg KLH intradermally; skin tests: 1 and 10 μg KLH intradermally; minimal and maximal diameters of induration measured after 24 and 48 h. ND = Not done.

Results

The skin, lymphocyte stimulation, and passive hemagglutination tests were negative in all subjects before immunization. The immunizing intradermal injection of 100 μg KLH produced some local irritation (itching, erythema) in nine subjects and none at all in three after 8 h to 6 days. All subjects showed some infiltration at the site of injection which was maximal after 3–8 days and lasted up to 2 weeks. Two subjects also had slight tenderness of local lymph nodes for a few days.

Skin tests done 1 and 2 weeks after immunization were positive in eleven subjects and negative in one (table I). The latter remained negative when retested 3 and 4 weeks after immunization. She is a healthy 20-year-old girl with no other evidence of deficient immune mechanism. Skin tests done with 1 μg KLH frequently gave negative results in subjects who were skin test-positive with 10 μg.

The peripheral blood lymphocytes of the eleven subjects with positive skin test included a small percentage of specifically sensitized lymphocytes as shown by the lymphocyte blastogenesis test (table II).

Table II. In vitro lymphocyte blastogenesis after stimulation with 10 and 100 μg KLH in individuals immunized with a single intradermal dose of 100 μg KLH[1]

Subjects	Before immunization		7 days after		14 days after	
	10 μg	100 μg	10 μg	100 μg	10 μg	100 μg
1	0.4	0.4	0.5	0.5	–	–
2	0.4	0.6	0.5	0.5	–	–
3	0.2	0.2	0.2	0.2	1.0	–
4	0.3	0.4	1.2	1.2	–	–
5	0.4	0.4	4.2	2.2	0.7	0.8
6	0.4	0.5	0.8	0.9	2.5	14.1
7	0.3	0.2	4.3	2.7	1.3	1.2
8	0.3	0.2	0.2	0.5	2.8	16.1
9	0.3	0.3	1.4	3.2	4.5	3.7
10	0.8	0.7	1.5	3.0	15.3	14.6
11	0.7	0.4	–	–	1.2	2.6
12	0.2	0.2	–	–	3.8	4.8
Mean	0.4	0.4	1.5	1.5	3.7	7.2

[1] Results in percentage of maximal stimulation with phytohemagglutinin.

The passive hemagglutination titers 2–4 weeks after immunization were positive in all subjects with positive skin test (table IV). The titers were generally low (1:8 to 1:32) except in two subjects (1:128). No significant titer was noted 7 days after immunization, but a rise was noted after 2–4 weeks. The hyperimmunized serum (positive control) gave exceedingly high titers (1:800 to 1:3,200). Precipitating antibodies (Ouchterlony technique) could be demonstrated only in the serum of the hyperimmunized (positive control) patient in dilutions of up to 1:8.

Comments

Hemocyanins have been known for a long time to be potent and reliable antigens and have been used extensively in animal experiments [CAMPBELL *et al.*, 1963]. Our study shows that one of them, KLH, can be used effectively in humans. The single immunizing dose of 100 μg is very small; however, CURTIS *et al.* [1971] have shown that even smaller single doses (10, 1, or 0.1 μg) are sufficient to produce cutaneous sensitization, the smaller doses taking longer to do this (28 days after 0.1 μg KLH).

Therefore, the usual recommended dose of 5 mg seems unnecessary. It may be preferable for measuring humoral immune reactions with differentiation of primary (19S) and secondary (7S) reactions [SWANSON and SCHWARTZ, 1967; TURK and WATERS, 1969; WHO, 1969].

Preparation of the antigen is relatively simple, the only real difficulty occurring during Millipore filtration (pore size 0.2 μm) which was possible only with relatively diluted solutions of the antigen. Thus the volume of the immunizing dose of 100 μg was too large for a single intradermal injection and was distributed to five wheals of 0.2 ml each.

In this method of antigen preparation (pH 7.2) the KLH is present predominantly in the associated form with a sedimentation constant of 98S and a molecular weight of 6×10^6. This giant molecule is highly immunogenic [CAMPBELL *et al.*, 1963; SWANSON and SCHWARTZ, 1967]. On the other hand, GREEN and BORELLA [1971] have demonstrated that it is active as a nonspecific mitogen, an effect that was negligible if present at all in our study.

The immunization procedure itself was tolerated well by the subjects; there was only mild local itching and erythema (like a mosquito bite) but no systemic reaction. This contrasts favorably to the more extensively used DNCB which frequently gives unpleasant burn-like local skin reactions. Another disadvantage of DNCB is that it does not always produce a hypersensitive state. This leads us to suspect that with this agent the immunizing and toxic doses overlap. Repeated skin testing with KLH may eventually act as a booster; however, this is only of limited theoretical interest, since in practice we simply want to test whether a specific reaction can be elicited in the patient.

Another important point is the danger of untoward reactions on reexposure of the patient or immunizing physician to the antigen. This is a real problem with DNCB, which is widely used in laboratories (e.g. in photographic developers). Sensitization was actually observed in a number of immunologists who have worked with DNCB. The danger of natural reexposure to KLH is minimal, since the protein is obtained from a nonedible snail. Immunological cross-reactions of the hemocyanins derived from *Megathura crenulata* (giant keyhole limpet) and *Limulus polyhemus* (horseshoe crab) do not occur according to MALLEY *et al.* [1971]. Besides, the hemocyanins undergo degradation in the gastrointestinal tract, so that they have to be given parenterally to be immunogenic. Thus the risks of reactions after ingestion of shellfish, crayfish, oyster, crab, and shrimp in persons sensitized with KLH is improbable.

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