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THE HUMAN SECONDARY IMMUNE RESPONSE TO KEYHOLE LIMPET HAEMOCYANIN

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SUMMARY

The human secondary immune response to the protein antigen Keyhole limpet haemocyanin (KLH) was studied in thirteen normal adults. For secondary immunization and for measurement of delayed hypersensitivity, 100 μg of KLH was given intradermally to each individual between 81 and 375 days after primary immunization. Immunity was assessed by delayed hypersensitivity skin testing and measurement of antibody formation and KLH stimulated *in vitro* lymphocyte blastogenesis. Twelve of the thirteen subjects had positive KLH delayed hypersensitivity reactions. Prior to secondary immunization all thirteen had circulating lymphocytes responsive *in vitro* to KLH and all had circulating haemagglutinating antibody. After secondary immunization there was no further increase in the mean KLH-stimulated blastogenesis while the KLH haemagglutinin titre rose due to increased 7S antibody formation. Primary immunization with small doses of KLH was as effective in inducing immunological memory and a subsequent secondary response as immunization with large doses of the antigen.

INTRODUCTION

In this investigation, the human secondary immune response has been studied using the protein antigen Keyhole limpet haemocyanin (KLH) (Swanson & Schwartz, 1967; Salvaggio, Castro-Murello & Kundur, 1969; Turk & Waters, 1969; Curtis *et al.*, 1970). The objectives were (a) to compare the primary and secondary immune response of normal adults to KLH, (b) to document the kinetics of the antibody response and of the *in vitro* lymphocyte blastogenic response after secondary immunization and (c) to investigate the relationship between the duration of immunological memory, the capacity to produce a secondary immune response and amount of antigen used for primary immunization.

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MATERIALS AND METHODS

Thirteen normal adults who had been immunized with KLH during studies of the human primary immune response (Curtis *et al.*, 1970; Curtis *et al.*, 1971) participated in the present investigation. Primary immunization consisted of a single intradermal or subcutaneous injection of 1–5000 μg of KLH and a 100 μg skin test 7–21 days later. The secondary immune response was studied 81–375 days after the application of this skin test (Table 1).

TABLE 1. KLH Delayed hypersensitivity responses

Subject No.	Initial dose of KLH (μg)	First skin test*	Second skin test	Interval between skin tests (days)
1	5000	7·7†	6·0†	81
2	5000	11·0	9·0	99
3	5000	7·5	6·0	318
4	5000	5·5	9·0	341
5	5000	7·5	7·5	375
6	100	0·0	0·0‡	322
7	100	23·5	15·0	335
8	100	10·5	9·0	341
9	10	5·5	6·5	201
10	10	8·0	5·0	217
11	10	11·5	13·5	217
12	1	5·5	6·5	138
13	1	0·0	18·0	201
Mean ± S.D.		8·0 ± 5·7	8·7 ± 4·6	

* Applied 7–21 days after primary immunization.
† Mean diameter in mm at 24 hr.
‡ Third skin test (see text).

After blood was drawn for KLH antibody determinations and for lymphocyte cultures, 100 μg of KLH in 0·1 ml of normal saline was injected intradermally on the volar aspect of the forearm. This served both as a skin test for KLH delayed hypersensitivity and for secondary immunization. The induration at the skin test sites was measured (Curtis *et al.*, 1970). Additional blood specimens were obtained 7, 14, 21, 28 and 42 days after secondary immunization with KLH.

KLH was prepared from live Keyhole limpets (*Megathura crenulata*) (Pacific Biomarine Co., Venice, California) as described by Campbell *et al.* (1964) and Curtis *et al.* (1970).

Lymphocyte blastogenesis

The methods of lymphocyte culture and measurement of blastogenesis by ^{3}H-thymidine incorporation have been described in detail (Curtis *et al.*, 1970). A set of cultures for each individual consisted of an unstimulated control and cultures containing 0·05 ml PHA (phytohaemagglutinin-M, Difco Laboratories, Detroit, Michigan), 0·1 ml SLO (Streptolysin-O, Difco Laboratories), 0·1 ml Streptokinase-Streptodornase (SK-SD) (Varidase, Lederle Laboratories, Pearl River, New York) and the following amounts of KLH per

millilitre of culture, 0·200, 0·100 and 0·010 mg. After incubation for 5 days, the lymphocyte cultures were pulse-labelled for 3 hr with 2 μCi of ^{3}H-thymidine (specific activity of 6·7 Ci per mM, Schwartz Bio Research, Orangeburg, New York). The amount of ^{3}H-thymidine incorporated by the acid-insoluble fraction of the lymphocytes was measured by liquid scintillation counting and expressed as counts per minute per 10^6 lymphocytes.

KLH haemagglutinin titres

KLH antibody titres were determined by the haemagglutination of KLH-coated tanned red blood cells (Swanson & Schwartz, 1967; Curtis *et al.*, 1970). 7S antibody was estimated following the treatment of sera with 0·1 M 2-mercaptoethanol (Deutsch & Morrow, 1957).

On several occasions, 7S and 19S antibodies were separated by sucrose density gradient centrifugation. The test sera were diluted 1:2 with phosphate buffer (pH 7·0) and 0·4 ml was placed on a 10–40% sucrose gradient The specimens were centrifuged for 6 hr at 60,000 rev/min in a Beckman L-4 Preparative Ultracentrifuge (Beckman Scientific Corporation, Fullerton, California). Fractions were collected manually (12 drops per tube). The type of antibody in each fraction was determined by radial immunodiffusion against IgG and IgM (Hyland Laboratories, Los Angeles, California). Fractions containing IgG or IgM were pooled and dialysed against normal saline for 24 hr in the cold. The volume was then condensed to 0·2 ml and the KLH haemagglutinin titre determined.

RESULTS

In Table 1, the KLH delayed hypersensitivity responses 7–21 days after primary immunization are compared with those done 81–375 days later. The first and second skin tests were quantitatively similar. The size of the second skin tests was not influenced by the length of the interval between the two skin tests or by the amount of KLH used for primary immunization. One patient (No. 9 in Table 1) was KLH skin test negative 7 days after primary immunization but had KLH delayed hypersensitivity when skin tested again 7 days later. A third skin test applied 322 days after the second skin test was again negative. This individual was in apparent good health, formed KLH antibody normally and had circulating lymphocytes responsive *in vitro* to PHA and various antigens including KLH.

Significantly increased KLH-stimulated lymphocyte blastogenesis was present as long as 375 days after the last exposure to KLH (Table 2) (Hersh & Brown, 1971). The net mean KLH-stimulated ^{3}H-thymidine incorporation immediately prior to secondary immunization was 1845 cpm per 10^6 lymphocytes. Despite considerable individual fluctuation there was no further increase in the mean KLH stimulated blastogenesis after secondary immunization. This is in contrast to the more than ten-fold increase in mean KLH stimulated lymphocyte blastogenesis that occurred after primary immunization with KLH (Curtis *et al.*, 1970). The magnitude of the KLH-stimulated blastogenesis during the secondary immune response was not influenced by the length of the interval between primary and secondary immunization or the amount of KLH used for primary immunization.

The lymphocyte blastogenic responses induced by PHA, SLO and SK-SD were similar during both the primary and secondary immune response studies. Immunization with KLH had no effect on the *in vitro* lymphocyte response to PHA or to the established antigens SLO and SK-SD.

The mean haemagglutinin titres after primary and secondary immunization with KLH are compared in Fig. 1. All thirteen subjects had KLH haemagglutinins of the 19S type present in their sera prior to secondary immunization. The persons whose primary immunization was with larger doses of KLH had a significantly higher mean 7S antibody titre before secondary immunization than did those whose primary immunization was with smaller doses of KLH ($P<0{\cdot}05$, Student's t-test). There were five persons who were given 5,000 μg or more of KLH and eight who were given 300 μg or less of KLH during the period of primary immunization and skin testing. The average interval between last exposure to KLH and secondary immunization was 243 days for the high dose group and 246 days for the low dose group. After secondary immunization with KLH, the mean increase in total

TABLE 2. KLH stimulated net ^{3}H-thymidine incorporation after secondary immunization with KLH

Subject No.	Primary immunization dose of KLH	Days after secondary immunization with KLH				
		0	7	14	28	42
1	5000	10233*	N.D.	6692	3237	1336
2	5000	2170	3246	6622	3342	3077
3	5000	814	460	477	N.D.	373
4	5000	1338	2570	721	3618	575
5	5000	3886	450	392	213	1213
6	100	2072	1048	N.D.	307	2232
7	100	8908	N.D.	16091	14483	6528
8	100	1028	379	2420	2430	2586
9	10	805	N.D.	1248	1179	1133
10	10	2302	N.D.	7764	2695	2508
11	10	557	N.D.	3371	3262	16839
12	1	726	2767	1260	441	4360
13	1	9168	N.D.	14121	14592	10857
Mean (Geometric)		1845	1430	2705	2039	2414

* cpm per 10^5 lymphocytes.
N.D. = not done.

antibody titre was 67% for those who had previously been exposed to high doses of KLH and 111% for the low dose group (Table 3). After secondary immunization with 100 μg of KLH, most of the increased antibody formation occurred during the first 7 days and consisted almost entirely of 7S antibody.

The predominant type of antibody in the sera immediately before secondary immunization was 2-mercaptoethanol sensitive. This antibody was considered to be 19S or IgM in type (Deutsch & Morrow, 1957). Sera from five patients were fractionated by sucrose density gradient centrifugation and the KLH haemagglutinin titre determined for the fractions containing 19S (IgM) and 7S (IgG) antibodies (Table 4). These studies indicated that treatment of the serum with 0·1 M 2-mercaptoethanol could be used to identify the classes of KLH antibodies present.

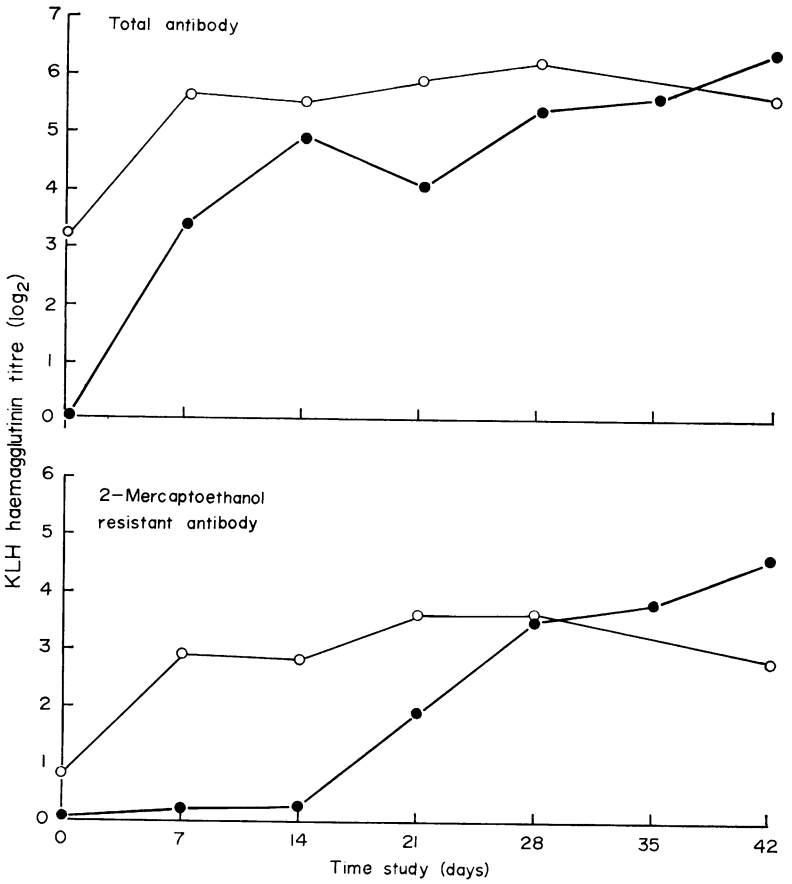

FIG 1. Mean haemagglutinin titres of thirteen normal adults after primary (●) and secondary (○) immunization with KLH.

TABLE 3. Influence of dose of KLH used for primary immunization on the antibody response after secondary challenge

Days after immunization	KLH antibody titre* (log_2)					
	0	7	14	21	28	42
Initial immunization > 5000 μg KLH (five subjects)						
Total	4·2±1·9	6·0±1·7	6·2±2·9	6·2±2·4	7·0±1·2	6·0±2·0
7S	1·8†±1·5	4·2±1·4	3·5±2·9	3·5±2·9	3·4±1·4	3·4±1·8
Initial immunization ≤ 300 μg KLH (eight subjects)						
Total	2·6±1·5	5·4±1·7	5·0±2·0	5·7±2·3	5·7±1·6	5·4±0·9
7S	0·1†±0·5	2·0±2·1	2·4±2·1	3·7±2·6	3·9±2·5	2·4±2·2
All subjects						
Total	3·2±1·6	5·6±1·7	5·5±2·3	5·9±2·0	6·2±2·3	5·6±1·4
7S	0·8±1·1	2·9±2·2	2·8±2·3	3·6±2·4	3·6±2·0	2·8±2·0

* Mean ± standard deviation.

† Difference significant ($P < 0·05$ and $P < 0·02$).

TABLE 4. Comparison of KLH antibody titres after separation of antibody classes by sucrose density gradient centrifugation and after treatment of sera with 0·1 M 2-mercaptoethanol

Subject No.	Days after secondary immunization with KLH					
	0	7	14	21	38	42
1	3/2* (3/1)†	—	3/3 (4/3)	—	5/6 (4/4)	—
2	4/0 (5/0)	—	—	—	6/8 (8/8)	5/7 (8/6)
3	2/0 (2/1)	—	3/2 (4/0)	—	6/8 (4/3)	—
4	3/2 (4/0)	—	—	—	—	4/2 (2/1)
5	—	3/7 (3/5)	2/5 (2/5)	4/3 (3/5)	—	—

* IgM titre/IgG titre determined on fractions separated by sucrose density gradient.

† IgM titre/IgG titre as calculated from total antibody titre and 2-mercaptoethanol resistant antibody titre (IgG).

DISCUSSION

Primary immunization with small doses of KLH induced an immunological memory state capable of inducing a secondary response similar to that observed following primary immunization with large doses of antigen. The amount of prior exposure to KLH did not quantitatively affect the delayed hypersensitivity responses or the magnitude of the KLH-stimulated lymphocyte blastogenesis 2–13 months later (Mote & Jones, 1963; Maurer, 1966; Stewart, 1968). Although subjects immunized initially with smaller doses of KLH had lower antibody titres before secondary immunization, these persons had a relatively greater rise in antibody titre after secondary immunization (Table 3). These observations suggest that small doses of antigen may be as effective as large doses in inducing a state of immunity capable of producing a strong secondary type response.

In the present investigation a further increase of KLH-stimulated blast transformation after secondary immunization was not observed. Following BCG vaccination in man, an increased number of cultured peripheral blood lymphocytes responding to PPD by blast transformation has been reported (Hughes, 1968). Studies of the secondary antibody response in animals have indicated that to elicit a secondary response the antigen dose must be equal to or greater than the dose used for primary immunization (Nossal, Austin & Ada, 1965; Smith *et al.*, 1970). Similarly, a higher threshold dose may exist for the initiation of further increase in antigen-specific blastogenesis by the lymphocytes of an immune individual.

The persistence of relatively large amounts of 19S antibody in the serum for months after primary immunization has not been reported frequently (Uhr *et al.*, 1967; Gleich *et al.*, 1966; Borel, Fauconnet & Miescher, 1964). It has been suggested that persistent 19S antibodies represent 'natural' antibodies (Boyden, 1966; Rowley & MacKay, 1969) or are due to exposure to cross-reacting antigens (Rowley, 1970). However, continued formation of 19S antibody may be characteristic of certain types of antigen or related to the dose and route of administration of the antigen. In the present study primary immunization with either a large or a small dose of KLH was associated with 19S antibody formation but only a large dose of antigen induced persistent 7S antibody formation.

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