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THE HUMAN PRIMARY IMMUNE RESPONSE TO KEYHOLE LIMPET HAEMOCYANIN: INTERRELATIONSHIPS OF DELAYED HYPERSENSITIVITY, ANTIBODY RESPONSE AND *IN VITRO* BLAST TRANSFORMATION

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SUMMARY

The immune response to a protein antigen, keyhole limpet haemocyanin, was studied in fourteen normal subjects and twenty-one patients with solid tumours. Immunological responsiveness was assessed by intracutaneous skin testing, by haemagglutinin titres and by *in vitro* blast transformation. No significant difference was found in the kinetics or magnitude of the immune response among subjects immunized with 0·01, 0·10, or 5·0 mg. Delayed hypersensitivity to keyhole limpet haemocyanin developed in thirty-two of thirty-four skin tested; a positive antibody titre occurred in all; and thirty-one of thirty-five had positive *in vitro* responses. Patients in good general condition (Group 1) had significantly greater delayed hypersensitivity and antibody responses than the normals but similar *in vitro* responses. All immunological parameters were depressed in the patients with advanced neoplastic disease (Group 2).

Although only skin test positive subjects had positive *in vitro* responses, no direct correlation was found between the degree of delayed hypersensitivity and the degree of *in vitro* blast transformation. Excellent correlation was demonstrated, however, between the *in vitro* response and the haemagglutinin titre (correlation coefficient +0·52, standard error ±0·09).

INTRODUCTION

Characterization of the immune response to specific antigens has become important in a variety of clinical situations. These include organ transplantation, tumour immunity and autoimmune states. To date, the cellular and humoral responses of normal adults to a primary antigen have not been well delineated. This has been due to the difficulty of ensuring that the response elicited after antigenic stimulation is a primary one and not a secondary or anamnestic response.

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Keyhole limpet haemocyanin (KLH) is a primary antigen suitable for study of the human immune response. KLH is a copper-containing protein which can be obtained in a pure state from the haemolymph of the keyhole limpet, *Megathura crenulata* (Campbell *et al.*, 1964). Since this is an inedible marine mollusc, prior human contact and sensitization is unlikely. In rats, minks and rabbits, Dixon, Jacot-Guillormod & McConahey (1967) have shown that KLH produces a prompt humoral antibody response while others (Vischer, Stastny & Ziff, 1967) have demonstrated a vigorous *in vitro* response by lymphoid cells from sensitized rabbits. Similarly, in humans early and persistent development of cellular and humoral immunity has been reported by Swanson & Schwartz (1967). To date, there has been no report of systemic toxicity or adverse local reactions to KLH.

The objectives of the current study were: (1) to demonstrate that KLH can induce antibody, delayed hypersensitivity and *in vitro* lymphocyte responses; (2) to document the kinetics of the development of these reactions after immunization, and (3) to study the interrelationships among antibody production, delayed hypersensitivity and *in vitro* blastogenesis. Also, some preliminary clinical studies are outlined illustrating how KLH can be used to evaluate immunological deficiency states.

METHODS AND MATERIALS

Immunized subjects

There were fourteen normal subjects who were volunteers from the medical staff. Their ages ranged from 27 to 64 years and the mean age was 41 years.

Twenty-one patients of the University of Texas M. D. Anderson Hospital and Tumor Institute were studied. All had a histologically proven diagnosis of a malignant solid tumour. Nine patients (Group 1) were ambulatory, leading relatively normal lives and had no detectable residual tumour or apparently localized disease. In this group, there were seven cases of malignant melanoma, one of adenocarcinoma of unknown origin and one of carcinoma of the nasopharynx. Their ages ranged from 39 to 67 years and the mean age was 54 years.

The remaining twelve patients, called Group 2, had advanced symptomatic disease. Nine had malignant melanoma and one each had bronchogenic carcinoma, synovial sarcoma and adenocarcinoma of unknown origin. The age range was 29–67 years and the mean age was 46 years. Four of these twelve patients (Nos: 27, 30, 32, and 33) had received chemotherapy in the month prior to immunization. The white blood cell counts and differentials, the extent of disease, and the general clinical condition of these four were similar to those of untreated members of Group 2. No patient was treated with anti-neoplastic drugs for 1 week before and 1 week after immunization with KLH. In the later stages of their study, all twelve patients received intermittent cancer chemotherapy.

Preparation of keyhole limpet haemocyanin (KLH)

Live keyhole limpets were obtained from the Pacific Biomarine Supply Company, Venice, California, and the KLH was prepared according to the method of Campbell *et al.* (1964). The final preparation was adjusted to 20 mg of protein nitrogen per millilitre and was stored under toluene at 4°C. The pH was consistently below 7 indicating that the KLH was mainly in the associated form (molecular weight $7{\cdot}5 \times 10^6$) (Bartel & Campbell, 1959). The material

used for immunization and skin testing contained 1 : 10,000 merthiolate while that used in tissue culture work contained 100 mg of penicillin and 100 μg of dihydrostreptomycin per millilitre.

Immunization and skin testing

Seventeen persons including six normal subjects were immunized with 5·0 mg of KLH subcutaneously in the deltoid region. Ten patients and four normals were given an initial dose of 0·10 mg, intradermally, while four normal persons were immunized with 0·01 mg, intradermally.

Skin testing was done with solutions containing 0·10, 0·01 or 0·001 mg of KLH per 0·1 ml of saline. Thirty-four persons were skin tested with 0·10 mg. In addition, twenty-three of these subjects were simultaneously skin tested with 0·01 mg of KLH and twelve with 0·001 mg. All skin tests were placed on the volar aspects of the forearm and read at 24 and 48 hr by the same observer (J.E.C.) The diameter of induration in millimeters was measured for two diameters at right angles and the mean of these measurements recorded.

Methods of tissue culture

The tissue culture methods were modified from Oppenheim, Whang & Frei (1965). The subjects were bled with plastic syringes and the blood defibrinated in sterile Erlenmeyer flasks with 3 mm glass beads (Scientific Products, Evanston, Illinois). After a sample was removed to prepare a serum specimen, a one-tenth volume of 4% w/v dextran (Pharmachem Corporation, Bethlehem, Pennsylvania, molecular weight 200,000) in saline was added. The red cells were sedimented at room temperature for 50 min in 20 × 200 mm glass tubes placed in the horizontal position. The leucocyte-rich plasma–dextran mixture was collected and the white blood cell count and differential determined.

Cultures were set up in vertical 13 × 10 mm Pyrex glass round bottom screw cap tubes. The culture volume, constant at 3 ml in all cultures was made up of 1 ml of autologous serum adjusted to contain 10^6 lymphocytes and 2 ml of Spinner modified Eagles Minimal Essential Medium (Hyland Laboratories, Los Angeles, California) supplemented with 20 mM/l of glutamine and a Penicillin–Streptomycin solution. Each set of cultures for an individual consisted of an unstimulated control culture and the following stimulated 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 (Varidase, Lederle Laboratories, Pearl River, New York), 0·1 ml vaccinia solution (Vaccinia, Dryvax-Wyeth, 1 : 100 dilution of standard vaccination suspension) and the following amounts of KLH per millilitre of culture, 0·200, 0·100, 0·010, 0·002, and 0·001 mg. All cultures were incubated for 5 days at 37°C in 5% carbon dioxide in air.

Measurement of [^{3}H]thymidine incorporation

To measure the rate of DNA synthesis of the cultured cells 2 mCi of [^{3}H]thymidine (New England Nuclear Corporation) having a specific activity of 6·7 Ci/mM were added on the 5th day of culture and the cells incubated for an additional 3 hr. The cultures were iced, the cells washed twice with cold saline, precipitated twice with 5% trichloracetic acid and the acid insoluble radioactivity processed for liquid scintillation counting in a standard manner. Quenching corrections were made when necessary. [^{3}H]thymidine incorporation was ex-

pressed as counts/min/10^6 lymphocytes. The appropriate unstimulated control was subtracted from the stimulated cultures to estimate the net incorporation. A positive response occurred when the net incorporation of the KLH stimulated culture increased three-fold over the pre-immunization studies. This was arbitrarily determined when it was found that all of the normal subjects had an *in vitro* response greater than this. Evidence of an *in vitro* dose response was found in every case considered a positive response.

KLH haemagglutinin titres

The tanned red cell haemagglutinin test was performed according to the method of Stavitsky (1954). Washed sheep red blood cells (SRBC) were mixed with an equal volume of a dilute tannic acid solution (0·005%), washed again and made up to a 5% solution in phosphate-buffered saline (PBS). After dilution, 1 part to 4 with PBS (pH 6·4), 2 mg/ml of KLH was added to the SRBC suspension and the mixture incubated for 10 min. After washing the KLH-coated SRBC were resuspended as a 3–5% suspension in 0·2% gelatin in saline. Control SRBC suspensions lacking KLH were prepared simultaneously. The sera to be tested were absorbed with SRBC, decomplemented and serial dilutions of 0·10 ml were prepared in 10 × 100 glass tubes. An equal volume of KLH-coated SRBC suspension was added to each tube and the amount of haemagglutination was determined microscopically 15–17 hr later. The results were expressed in terms of the reciprocal of the highest dilution of sera which resulted in haemagglutination.

For each serum specimen the 7S antibody titre was determined as 2-mercaptoethanol resistant (2-MER) antibody (Deutsch & Morrow, 1957; Dixon, Jacot-Guillormod & McConahey, 1966). The amount of antibody sensitive to 2-mercaptoethanol (19S) was calculated by subtracting the 7S antibody titre from the total antibody titre.

RESULTS

Delayed hypersensitivity

All fourteen normal subjects immunized with KLH developed typical delayed hypersensitivity (Table 1). Four of the five skin tested between 5 and 7 days after immunization were positive. The one negative converted to positive when retested 18 days later. The three first tested at 14 days had positive intradermal tests as did the five first tested at 21 or more days post-immunization.

Ten of the eleven cancer patients immunized with 5 mg of KLH were subsequently skin tested with 0·10 mg of KLH from 5 to 28 days after immunization. All had positive *in vivo* responses on first testing. Ten patients were immunized with 0·10 mg and six of these ten demonstrated a positive delayed hypersensitivity response when first tested. The KLH skin tests were repeated in two instances and found to be positive 8 and 11 days later. The other two patients were very ill and were anergic to a number of skin test antigens.

The mean diameter of induration produced by the 0·10 mg KLH skin test dose in the normals was 10·3 mm (Table 1). The mean response in the Group 1 patients was 15·7 mm which was significantly different from that of the normal subjects at the 5% level ($\dagger = 2{\cdot}358$). Group 2 patients had a mean skin test response which was smaller than normal although the difference was not statistically significant. Comparing the Group 2 patients with the Group 1 patients, the delayed hypersensitivity response was significantly less in the former ($P \leqslant 0{\cdot}01$, $\dagger = 3.666$).

TABLE 1. KLH delayed hypersensitivity response

Normal subjects					Patients: Group 1				Patients: Group 2			
			Results of intradermal skin test‡ with:				Results of intradermal skin test‡ with:				Results of intradermal skin test‡ with:	
No.	Dose of KLH (mg)	Time of testing (days)	0·01 mg	0·10 mg	No.	Dose of KLH (mg)	Time of testing (days)	0·01 mg	0·10 mg	No.	Dose of KLH (mg)	Time of testing (days)	0·01 mg	0·10 mg
1	5·0*	5	ND§	10.0	15	5·0	7	ND	25·0	24	5.0	7	0	8·0
1		61	5·5	10·0	16	5·0	7	ND	24·0	25	5·0	14	ND	8·0
2	5·0	5	ND	7·5	17	5·0	5	5·0	10·0	26	5·0	7	0	9·5
3	5·0	5	ND	7·5	18	5·0	5	9·5	19·5	26		16	8·5	10·0
4	5·0	5	ND	7·0¶	19	5·0	5	5·0	8·5	27	0·10	7	ND	10·5
5	5·0	227	ND	12·0	20	5·0	28	ND	5·0	27		41	7·5	14·5
5		247	10·0	10·5	21	5·0	NT‖			28	0·10	12	0	5·5
6	5·0	48	ND	11·0	22	5·0	7	7·7	16·0	29	0·10	57	7·0	14·5
7	0·10†	14	ND	7·0	23	0·10	16	ND	13·3	30	0·10	6	0	0
8	0·10	14	ND	10·5	23		37	ND	20·0	30		17	6·5	9·0
9	0·10	7	0	0						31	0·10	52	12·5	15·5
9		25	0	5·0						32	0·10	48	13·5	15·5
10	0·10	14	10·0	23·5						33	0·10	5	0	0
11	0·01†	21	ND	8·0						34	0·10	6	0	0
12	0·01	21	ND	30·0						34		14	5·0	5·5
13	0·01	21	ND	11·0						35	0·10	39	0	0
14	0·01	21	ND	5·0										
Total No. of tests (No. of positive tests)			5(3)	17(16)				4(4)	9(9)				14(7)	16(12)
Mean ± SD (mm)			5·1	10·3 ± 6·9				6·8	15·7 ± 4·7				4·3 ± 4·9	8·0 ± 5·6
Median (mm)			5·5	10·0				6·3	16·0				2·5	8·5
Range (mm)			0–10·0	0–30·0				5·0–9·5	5·0–25·0				0–13·5	0–15·5

* Given subcutaneously.
† Given intradermally.
‡ Mean diameter of induration at 48 hr.
§ ND, Not done.
¶ Test read at 24 hr.
‖ Not tested.

TABLE 2. Effect of initial dose of KLH on delayed hypersensitivity response

Initial dose of KLH (mg)	Normals			Patients: Group 1		Patients: Group 2		All subjects		
	5·0	0·10	0·01	5·0	0·10	5·0	0·10	5·0	0·10	0·01
No. of subjects	6	4	4	7(8)†	1	3	9	16(17)†	14	4
Mean (mm)	9·2	10·2	13·5	15·4	13·5	8·5	6·8	11·8 ± 3·8*	12·9 ± 4·2*	13·5
Median (mm)	8·7	6·0	9·5	16·0	13·5	8·0	5·5	9·7	8·7	9·5
Range (mm)	7·0–12·0	0–23·5	5–30·0	5·0–25·0	—	8·0–9·5	0–15·5	5·0–25·0	0–23·5	0–30·0

* Standard deviation.

† One patient in Group 1 was never skin tested. Excluded from calculations.

The effect of the immunizing dose of KLH on the development of delayed hypersensitivity is shown in Table 2. The *in vivo* response was independent of the range of antigen dose employed.

In order to find the optimum dose of KLH for skin testing, twenty-three of the subjects were skin tested simultaneously with either two or three doses of the antigen (Table 3).

TABLE 3. KLH skin test response—intradermal dose of antigen

	Intradermal dose of KLH (μg)		
	1	10	100
No. of patients	12	23	23
No. of patients with positive tests	2	14	18
Mean ± SD (mm)	0·1	4·9 ± 4·5*	9·1 ± 6·6*
Median (mm)	0	5·0	9·5
Range (mm)	0–5·0	0–13·5	0–23·5

* $P < 0{\cdot}02$ Student's t-test ($r = 2{\cdot}530$).

Eighteen of the twenty-three skin tested with 0·10 mg were positive compared to fourteen of twenty-three tested with 0·01 mg. The 0·001-mg intradermal dose of KLH was the least effective producing only two positives in twelve tests. The magnitude of the skin response was also dose dependent. The reactions elicited with 0·10 mg are significantly larger than those produced by 0·01 mg of KLH intradermally.

Lymphocyte blastogenesis

The lymphocytes of all fourteen normal subjects developed a positive *in vitro* response to KLH (Fig. 1). The pre-immunization studies revealed a slight but definite and dose-dependent *in vitro* response to this antigen. Considerable variation among individuals is evident from Fig. 1 but for any one subject the response followed the general pattern. By 7 days post-immunization the mean [^{3}H]thymidine response was 3616 count/min/10^6 lymphocytes compared with 463 counts/min/10^6 lymphocytes prior to KLH immunization ($P < 0{\cdot}05$). Twenty-eight days after immunization, the mean [^{3}H]thymidine uptake had increased ten-fold over the pre-immunization mean. After 28 days, the *in vitro* lymphocyte responses tended to plateau with wide fluctuations.

The character of the *in vitro* response to KLH by the lymphocytes of sensitized normal persons is again illustrated in Fig. 2 and the median response is seen to increase steadily with time. Over the same period the [^{3}H]thymidine incorporation by the PHA stimulated cultures showed only a small, statistically insignificant, increase. The median [^{3}H]thymidine uptake in the cultures stimulated with vaccinia, SLO or Varidase fluctuated widely but demonstrated no specific trend. Immunization with KLH had no effect on the *in vitro* response to the mitogen PHA or to the three antigens to which prior immunity existed.

The *in vitro* response to KLH by the lymphocytes of Group 1 patients was similar to that of the normal subjects (Fig. 3). The lymphocytes of eight patients in Group 2 developed positive *in vitro* responses to KLH. Compared with the normals and Group 1 patients the

onset of increased blastogenesis was delayed. The differences observed were not statistically significant. Two Group 2 patients whose lymphocytes did not develop an *in vitro* response to KLH also failed to have positive delayed hypersensitivity reactions.

The effect of the immunizing dose of KLH on the development of *in vitro* blastogenesis is illustrated in Fig. 4. The [^{3}H]thymidine incorporation was essentially the same whether the immunizing dose of KLH was 5 g, 0·10 or 0·01 mg.

The dose–response curves of the KLH stimulated lymphocyte cultures of the fourteen

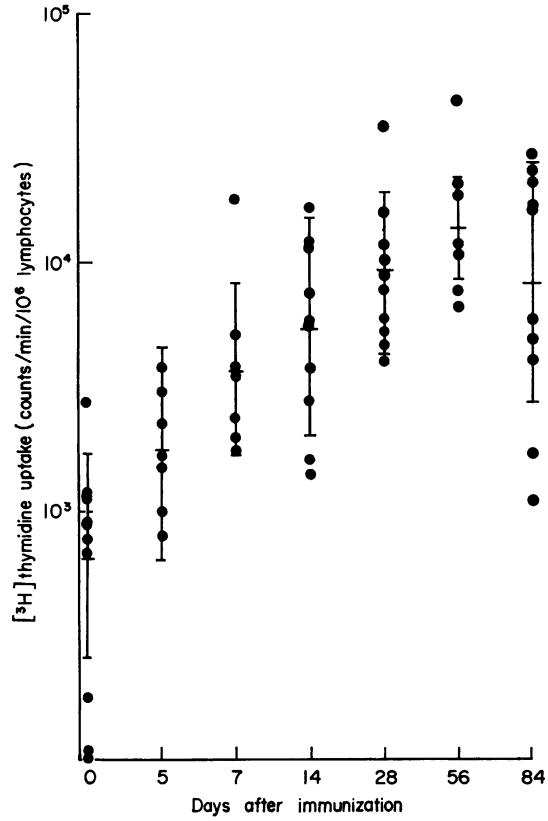

FIG. 1. *In vitro* lymphocyte blastogenic response of KLH stimulated cultures of fourteen normal subjects following immunization. Maximum [^{3}H]thymidine uptakes for each day are plotted. Horizontal bars indicate mean responses

normal subjects from day 0 to day 84 are presented in Fig. 5. At higher *in vitro* doses of KLH the response levelled off but showed no evidence of an inhibitory or toxic effect of the antigen. Maximum [^{3}H]thymidine uptake was obtained with an *in vitro* dose equal to or greater than 0·100 mg/ml of culture. Doses less than 0·010 mg/ml did not induce consistently satisfactory responses.

Antibody formation

The sera of all fourteen normal persons contained 19S KLH antibody by 7 days post-immunization (Fig. 6). The peak 19S antibody titre occurred between 14 and 21 days. The

onset of 7S antibody formation was more variable with only eight of the fourteen serum specimens obtained 28 days after immunization yielding positive titres for 7S antibody. 7S antibody was detectable in the sera of all the normal subjects by 56 days. The rate of onset of antibody production and the magnitude of the response was not related to the immunizing dose of KLH employed.

By 7 days post-immunization, the sera of all Group 1 patients had positive KLH antibody titres (Fig. 7). Comparison of the mean haemagglutinin titres of the normal subjects and the Group 1 patients demonstrated an earlier peak of 19S antibody production among the latter as well as an earlier onset of 7S antibody formation. In contrast, the antibody titres of the

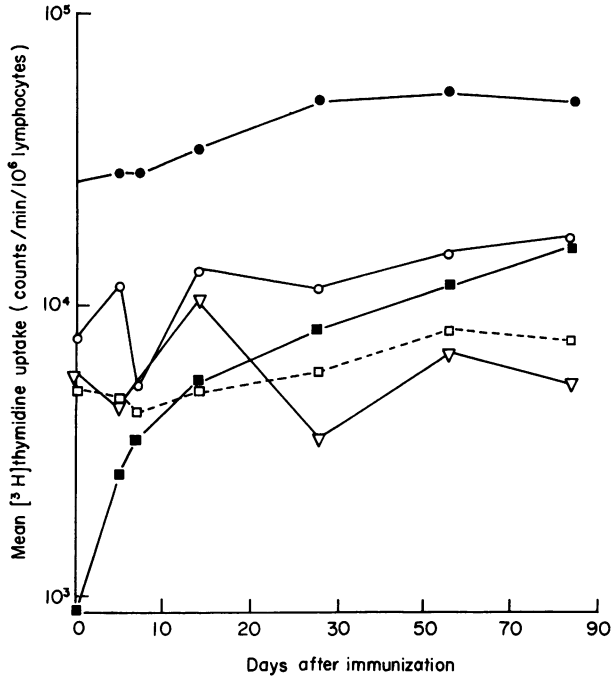

FIG. 2. *In vitro* lymphocyte blastogenic response of PHA and antigen stimulated cultures in fourteen normal subjects following KLH immunization. Median [H³]thymidine uptakes for the group are plotted. The steadily increasing response of KLH stimulated cultures with time after immunization is contrasted with the absence of specific trends in cultures stimulated with PHA and established antigens. ●, PHA; ○, SLO; ■, KLH; □, POX; ▽, VAR.

sera of the Group 2 patients were lower than those of the normals and there was a tendency for the antibody response to fall off by 28 days post-immunization (Fig. 8). The 7S KLH antibody response was relatively more depressed in this group of patients than was the 19S antibody response.

Correlation of immunological parameters

Delayed hypersensitivity and lymphocyte blastogenesis. Positive *in vivo* and *in vitro* responses to KLH tended to occur together. Only the lymphocytes of skin test-positive

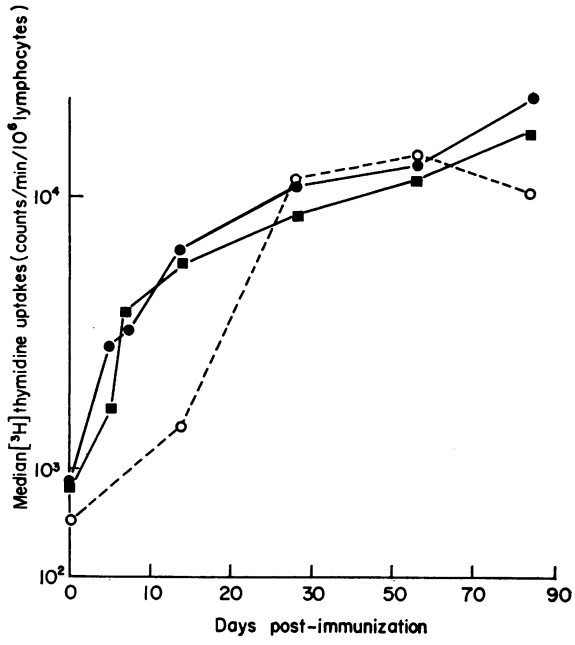

FIG. 3. Comparison of *in vitro* lymphocyte blastogenesis of KLH stimulated cultures of normal subjects (●) and patients (■, Group 1; ○, Group 2) following immunization. Despite early lower [³H]thymidine uptakes by Group 2 patients, no statistical difference was found among the three groups in their *in vitro* response.

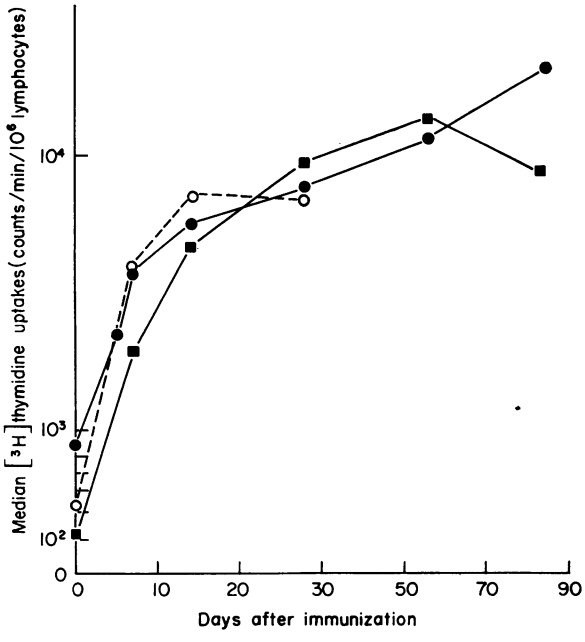

FIG. 4. Demonstrates lack of effect of the immunizing dose on *in vitro* lymphocyte blastogenesis in KLH stimulated cultures of fourteen normal subjects. ○, 0·01 mg; ●, 0·1 mg; ■, 5 mg.

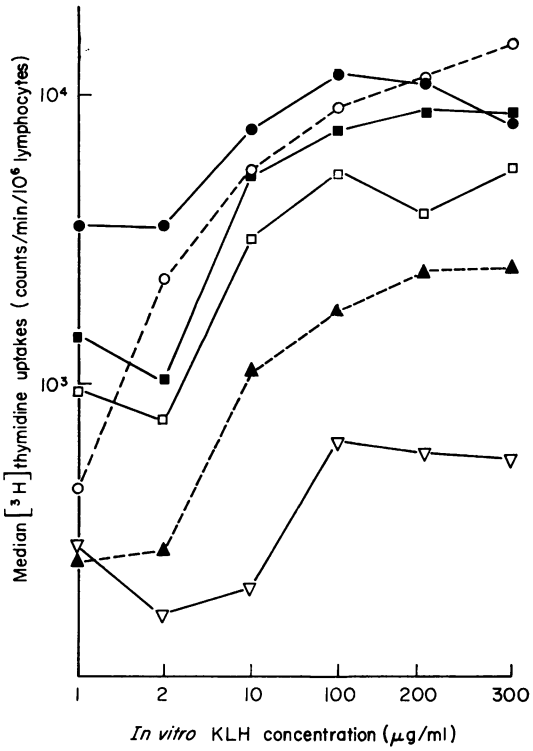

FIG. 5. KLH *in vitro* dose-response curves in fourteen normal subjects. The median [^{3}H]thymidine uptakes for the group for each day and for each *in vitro* dose of KLH are plotted. There was increased *in vitro* responsiveness for the first 28 days after which it tended to plateau. ▽, 0 days; ▲, 7 days; □, 14 days; ■, 28 days; ●, 56 days; ○, 84 days.

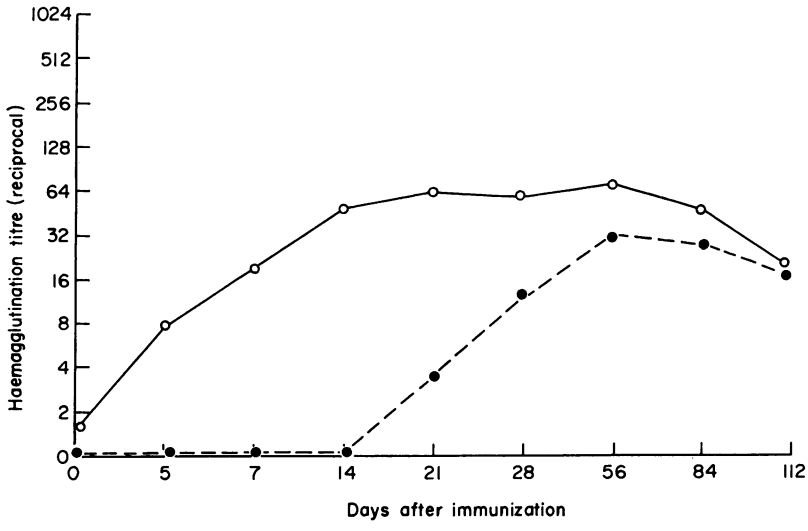

FIG. 6. Mean total and 7S antibody response of fourteen normal subjects after KLH immunization. ○, Total antibody; ●, 2-ME resistant antibody.

subjects responded *in vitro* although two persons with negative lymphocyte blastogenesis had positive skin tests. Among all the subjects studied, there were thirty-eight paired observations of the delayed hypersensitivity and the *in vitro* response. When the delayed hypersensitivity responses were divided into four groups according to increasing diameter of induration, the [^{3}H]thymidine uptakes were usually higher when the skin test reaction was

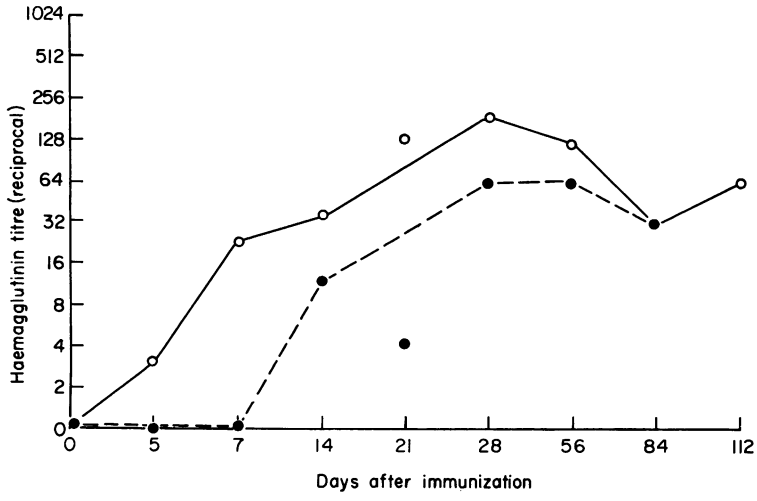

FIG. 7. Mean total and 7S antibody response of Group 1 patients after KLH immunization. ○, Total antibody; ●, 2-ME resistant antibody.

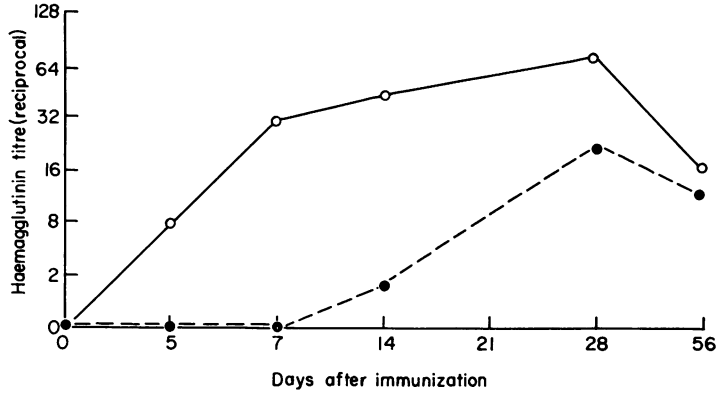

FIG. 8. Mean total and 7S antibody response of Group 2 patients after KLH immunization. ○, Total antibody; ●, 2-ME resistant antibody.

larger (Fig. 9). The relationship is not statistically significant when tested by an analysis of variance ($P > 5\%$). The mean [^{3}H]thymidine uptake for KLH stimulated cultures of those subjects with skin test diameters greater than 10 mm was 7690 counts/min/10^6 lymphocytes. When the skin test was less than or equal to 10 mm in diameter, the [^{3}H]thymidine uptake was 4070. Again, the difference is not significant at the 5% level.

Delayed hypersensitivity and antibody production. The relationship between delayed hypersensitivity and haemagglutinin titre is shown graphically in Fig. 10. There were thirty-eight paired observations drawn from both the normal subjects and the patient groups. For the four ranges of skin test response the median haemagglutinin titre for KLH was the same.

Lymphocyte blastogenesis and antibody production. Considering all subjects, there were 124 occasions on which the haemagglutinin titre and the *in vitro* response were determined simultaneously. The corresponding values of each have been plotted in Fig. 11. For the KLH

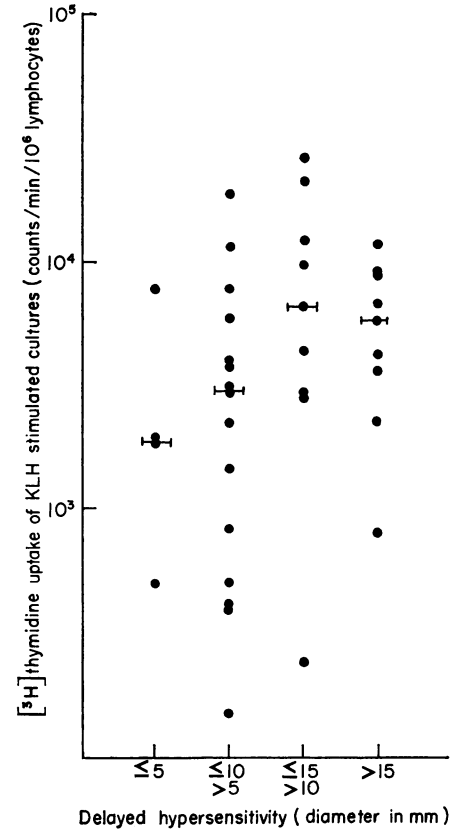

FIG. 9. Demonstrates relationship between the magnitude of KLH delayed hypersensitivity response and *in vitro* lymphocyte blastogenesis of KLH stimulated cultures. The horizontal bars indicate the median [^{3}H]thymidine uptake for that range of skin test response. No statistically significant trend was found.

antigen there was a considerable degree of association between these two immunological parameters. A tendency for a high *in vitro* response to occur with a high antibody titre and low [^{3}H]thymidine uptake to occur with low antibody response was observed. An analysis of variance indicated that the trend was significant at the 1% level. The correlation coefficient was $+0{\cdot}52$ with a standard error of $\pm 0{\cdot}09$.

DISCUSSION

A number of bacterial products are available for use as primary antigens in studying the human immune response. Vaccination with BCG and the vole bacillus are known to induce delayed hypersensitivity in 90% of children and adolescents who before immunization were skin test negative to 100 units of old tuberculin (Medical Research Council, 1959). Subclinical infection with atypical mycobacteria is common in certain geographical areas, and since these organisms cross-react with one another as well as *Mycobacterium tuberculosis* (Smith, 1967), mycobacteria may not be suitable primary antigens. In certain areas where

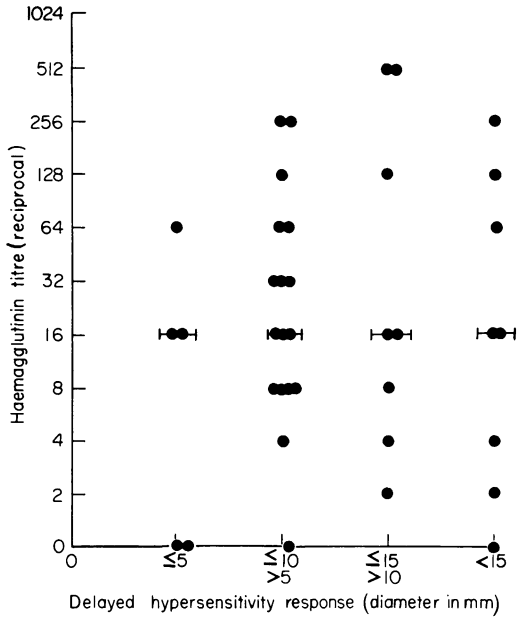

FIG. 10. Demonstrates the relationship between the magnitude of the KLH delayed hypersensitivity response and the KLH haemagglutinin titre. The horizontal bars indicate the median haemagglutinin titre for each range of skin test response.

the incidence of infection is low, Landy (1954), has suggested using the Vi antigen for primary immunization. Uhr (1964) found in his studies that bacteriophage ΦX174 gave excellent antibody responses but did not report on delayed hypersensitivity. Vaccines prepared from *Pasteurella talarensis* have been used as antigens. Using such a vaccine, Levin, Landy & Frei (1964) found positive delayed hypersensitivity in all four control patients 14 days after primary immunization. The problem of cross-reaction is inherent in the use of any microbial product and the immune experience of man is so heterogeneous that prior exposure can never be excluded with finality.

Contact hypersensitivity has been extensively studied with dinitrochlorobenzene (DNCB) (Kligman & Epstein, 1959; Waldorf, Wilkens & Decier, 1968) and to a lesser extent with other irritants such as pentadecyl catechol (Epstein & Kligman, 1957). Work with hapten–protein conjugates by Benacerraf & Gell (1959), Gell & Benacerraf (1961), and Salvin & Smith

(1960) has demonstrated a close relationship between contact hypersensitivity and delayed hypersensitivity. In addition to producing contact hypersensitivity in over 90% of healthy adults, DNCB, and similar chemicals induced brisk antibody responses (Kligman & Epstein, 1959; Waldorf *et al.*, 1968). Recently, conjugates of picryl or dinitrophenol (DNP) and human serum albumin (HSA) have been used in human beings (Kantor & Bullock, 1966). These conjugates have been reported to produce good delayed hypersensitivity, antibody

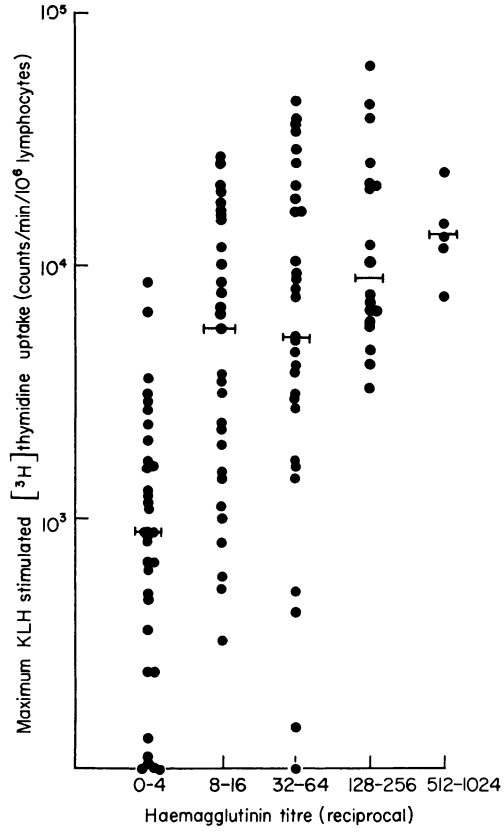

FIG. 11. Illustrates the relationship between the KLH antibody titre and the corresponding *in vitro* blastogenic response of KLH stimulated lymphocyte cultures. The horizontal bars indicate the median *in vitro* response for the various ranges of haemagglutinin titre. The association is statistically significant (correlation coefficient+0·52, SE+0·09).

response, and in addition, to stimulate *in vitro* lymphocyte transformation. Further evaluation is necessary as the administration of altered human serum proteins to human beings may not be without risk (Lerner, Glassock & Dixon, 1967).

Ethylene oxide-treated human serum has been used with some success by Maurer (Maurer, 1961) but is open to the same criticism as HSA conjugates.

Maurer and his co-workers (Maurer, Gerulat & Pinchuck, 1962) have used synthetic polymers of α-amino acids with a molecular weight of 35,000–50,000 to induce delayed hypersensitivity and antibody responses. Within 12 days, the delayed hypersensitivity response was positive in all twelve normal subjects immunized with a polymer containing the l-iso-

mers of glutamic acid, lysine, alanine and tyrosine. Positive skin tests to this and other polymers have been found to persist for as long as 6 years (Maurer, personal communication). Satisfactory acute phase antibody titres have been produced in nearly all normal subjects. Variable degrees of cross-reactivity with other amino acid polymers were found but otherwise they seemed to induce specific responses. Skin tests and antibody titres at the time of immunization were negative. Schlossman *et al.* (1966) have prepared α-DNP-oligo-L-lysine butylamides which they have exploited to characterize delayed and immediate hypersensitivity in animal systems. Like the α-amino acid polymers of Maurer these will likely prove useful in man.

In the current study, 94% of the thirty-four subjects skin tested had positive delayed hypersensitivity responses. Thirty-one of the thirty-five studied developed positive *in vitro* responses to KLH and all subjects had positive haemagglutination titres. The immune response to KLH was virtually complete by 14 days in normal subjects and cancer patients.

Evidence indicates that for man, KLH is a primary antigen. Seventeen persons with no prior exposure to the antigen were skin tested with 0·01 mg and no positive reactions were found. In addition, the pre-immunization antibody titres were negative. Specificity of the antigenic response is indicated by the dose response observed with the various concentrations of KLH used for skin testing and for the stimulation of lymphocyte blastogenesis (Table 3 and Fig. 5). KLH immunization did not alter the *in vitro* lymphocyte response to other antigens (Fig. 2). However, slightly positive responses before immunization were seen in the KLH stimulated lymphocyte cultures. This may indicate primary immunization *in vitro* or cross-reactivity with other antigens. Although KLH cross-reacts with some other haemocyanins (Dixon *et al.*, 1967) no cross-reactivity with other antigens used in man has been demonstrated (Malley, Saha & Halliday, 1965).

Neither the immunizing dose of KLH nor the route of administration altered either the kinetics or magnitude of the immune response. There was a 500-fold difference between the smallest and the largest initial dose. This is in keeping with the findings of Dixon and his colleagues (Dixon *et al.*, 1967) that in rabbits comparable antibody responses were induced by widely different antigen doses given by various routes. Despite the apparent excellent immunogenicity of KLH, a relatively large dose of 0·10 mg was required to consistently elicit a positive skin-test response indicating that the delayed hypersensitivity reaction required a high threshold to be detected.

Increased 7S and 19S antibody production and greater delayed hypersensitivity responses were noted among the Group 1 patients (Table 1 and Fig. 7). This may be due to selection of an unusual group of patients as the number studied is small. Six of the nine patients had a cutaneous malignant melanoma widely excised often with dissection of adjacent lymph node areas either during or a few days prior to the study period. The temporary impairment of the *in vitro* blastogenic response that has been reported to occur post-operatively (Riddle & Berenbaum, 1967) was not evident in this study. Possibly, increased immunogenicity is a feature of early or apparently localized malignant melanoma or of early and localized malignant tumours in general.

Impairment of the immune response is a regular feature of advanced malignant disease (Lamb *et al.*, 1962). The Group 2 patients received chemotherapy in short courses every 2 or 3 weeks. Several investigators have shown that with intensive chemotherapy there is suppression of the antibody response almost immediately but a prompt recovery within 24 hr of stopping treatment even in the face of falling lymphocyte counts (Hersh, Carbone &

Freireich, 1966). The four patients who had some chemotherapy before KLH immunization did not have responses differing from those who had no prior chemotherapy. Thus, the immunologic deficiency observed in the Group 2 cancer patients is related to their underlying disease rather than to the immunosuppressive effects of drugs.

In another clinical investigation Swanson & Schwartz (1967) immunized with 5 mg of KLH subcutaneously ten normal persons and twenty patients with non-neoplastic diseases who were receiving continuous treatment with azathioprine. The normal subjects developed delayed hypersensitivity by 5 days and had antibody responses similar to the normal subjects in the present study. In the patient group, nine of twenty never expressed delayed hypersensitivity even after repeated skin testing over periods up to 198 days. In addition, antibody responses in the patients were delayed. Since the disease states found in this study are not associated with impaired immunity (Saslow *et al.*, 1959), the results reflect the immunosuppressive effect of continuously administered azathioprine (Levin *et al.*, 1964).

In the present study, the lymphocytes of immunized subjects with negative skin tests demonstrated little *in vitro* blastogenesis whereas the lymphocytes of sensitized subjects with positive cutaneous reactions manifested considerable lymphocyte blastogenesis. Despite this, no statistically significant correlation between the size of the skin-test response and the degree of *in vitro* blastogenesis was found. Similarly, no relationship of a quantitative nature could be established between the degree of delayed hypersensitivity and the height of the antibody titres. However, a good association was demonstrated between KLH haemagglutination titre and the magnitude of the [^{3}H]thymidine uptake.

McFarland & Heilman (1966) and Kerby (1968) found a quantitative relationship between the tuberculin test and the degree of *in vitro* lymphocyte blastogenesis in response to various tuberculins. In sarcoidosis impaired delayed hypersensitivity responses have been found to be associated with poor degrees of lymphocyte transformation (Hirschhorn *et al.*, 1964). Hersh & Oppenheim (1965) have demonstrated a parallel depression of delayed hypersensitivity and *in vitro* blastogenesis in patients with Hodgkin's disease that fluctuates with their clinical state. In addition, the lymphocytes from patients with congenital or acquired hypogammaglobulinaemia, who reject homografts normally and can develop delayed hypersensitivity responses, respond *in vitro* to both non-specific stimuli and to most specific antigens (Bradley & Oppenheim, 1967; Cooperband, Rosen & Kilbrick, 1968).

Although non-specific stimulation of lymphocytes *in vitro* with PHA and other mitogens does not result in immunoglobulin synthesis (Greaves & Roitt, 1968), stimulation of sensitized cells with specific antigen does induce antibody formation (Forbes, 1965; Lamvik, 1968).

There is then evidence that *in vitro* lymphocyte blastogenesis is related to both cellular and humoral immunity. The current study of the primary immune response in man supports this contention. The circulating small lymphocytes are the cells which undergo transformation *in vitro* (MacKinney, Stahlman & Brecher, 1962). These are also the cells bearing the information involved in immunological memory (Gowans & Uhr, 1966). Thus, lymphocyte blastogenesis is likely a measure of immunological memory and as such is related to both the cell-mediated and humoral mediated aspects of immunity.

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