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The Makari Skin Test and Immunocompetence in Bladder Cancer

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Summary. In two groups of bladder cancer patients with localised pathological stage P1 or P2 tumours, 21 with and 22 without recurrent disease, the results of the Makari skin test were compared with measurements of immunological competence, including delayed hypersensitivity skin reactions to microbial antigens, lymphoproliferative response to PHA, T cell numbers and serum immunoglobulins. Both groups of patients showed a reduction in immunological competence when compared with a control group of 20 patients of a similar age range with benign prostatic hyperplasia. However, comparison of these measurements in 21 Makari positive with 22 Makari negative patients did not demonstrate any significant differences. It is concluded that further studies are required to elucidate the mechanism of the Makari skin test.

Key words: Makari skin test - Bladder cancer - Immunocompetence.

INTRODUCTION

The Makari skin test is an in vivo technique (11, 12, 13) which has been used to detect bladder cancer (15, 16, 23). The antigens used for this test, when injected intradermally, may produce an immediate erythematous reaction (22) which may be observed in patients with neoplastic disease, as well as with other conditions, such as hepatic cirrhosis, ulcerative colitis, diverticulitis and peptic ulcer. In cases of bladder carcinoma, positive tests have been observed in 35/37 (95%) patients with primary tumour and in 60/81 (74%) with recurrent cancer (15), indicating that this test might be of value as an adjunct to cystoscopy.

If the Makari test is to be used as a clinical tool a more exact understanding of its mechanism could lead to further improvements in accuracy. The present study was therefore initiated to determine the relationship of this test to measurements of immunological competence in patients with previous clinical and pathological evidence of bladder cancer. Two groups of patients were studied: those with and those without recurrence as confirmed by cystoscopic assessment and histological examination. As far as possible, in all the patients studied, measurements of cell-mediated immune competence (delayed hypersensitivity skin test using 'recall' antigens, quantitation of peripheral blood thymus-derived (T) lymphocytes, and response of lymphocytes to phytohaemagglutinin) and humoral immunocompetence (level of serum immunoglubulins) were compared with the results of the Makari skin test.

METHODS

Patients from two centres, who had been previously treated for histologically confirmed bladder cancer and were admitted for routine review cystoscopy at 3 to 12 month intervals, were asked for their consent to undergo the tests. Only male patients were studied in order to exclude possible variations in immunological status due to sex differences. All tumours were pathological stage P1 or P2, as determined using the UICC (1963) or the WHO criteria described by Pugh (19, 24).

Three groups of patients were investigated:

Group 1: 21 patients with clinical and histological evidence of recurrent bladder cancer.

Group 2: 22 patients with no clinical evidence of recurrence at cystoscopy.

Group 3: A control series of 20 patients of a similar age range and with benign prostatic hypertrophy.

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Four of the first group and three of the second had previously been treated with radiotherapy or chemotherapy.

(1) Makari Skin Test. The antigens, described as tumour polysaccharidal substances (9, 21), prepared from mitochondrial fractions of neoplastic tissue derived from lung carcinoma, sarcoma and lymphoma, were supplied by Ormont Diagnostics Limited (Englewood, N. J., U. S. A.). The technique of testing was that described by Munson and Tee (15).

(2) Cell-Mediated-Immunocompetence. (a) Delayed hypersensitivity skin test.

The patients were given four 'recall' antigens, using 0.1 ml intradermal injections in the forearm (see Table 1). Positive reactions were measured 48 hours later; patients with no response to all four antigens were regarded as 'anergic'. Fifteen Group 1 and twelve Group 2 patients were tested.

(b) Lymphoproliferative response to phytohaemagglutinin (PHA). T-lymphocytic activity was measured by the uptake of tritiated thymidine into newly-synthesized DNA in lymphocytes cultured in the presence of PHA, using a modification of the technique of Paty and Hughes (18). The cells from 5 ml of heparinised blood were washed three times with culture medium (Waymouth's MB752/1) to remove serum, and triplicate cultures were prepared each containing 10% washed cells with either 10% autologous or 10% pooled AB serum and 0, 25, 50 or 100 µg PHA. The cultures were incubated at 37°C for 70 hours in an atmosphere of 5% CO_2 in air, and were then exposed for 2 hours to 1 µC/ml tritiated thymidine (5 Ci/mmol, Radiochemical Centre, Amersham, U.K.). DNA was precipitated by trichloroacetic acid before scintillation counting in a toluene-based scintillant.

The results were expressed as a test/control (T/C) ratio - the mean counts per minute in the PHA stimulated culture (the set of three counts showing the greatest stimulation was selected) divided by the mean counts per minute in the control unstimulated culture.

(c) Rosette assays. The numbers of T-lymphocytes were estimated by spontaneous rosette formation with sheep red cells. Mononuclear cells were separated from 10 ml of heparinised blood on a Ficoll-Triosil gradient and washed three times in buffered Hank's solution at pH 7.2. A million lymphocytes were mixed with 100 million sheep erythrocytes in 0.5 ml medium (Waymouth's MB752/1), incubated at 37°C for five minutes, centrifuged, and incubated at 4°C overnight. At least 400 lymphocytes were observed and the numbers forming rosettes (with 3 or more attached sheep erythrocytes) were counted. The number of T-lymphocytes was expressed as a proportion of the total number of lymphocytes in the peripheral blood.

(3) Humoral Immunocompetence. The serum immunoglobulins IgA, IgG and IgM were measured on radial immunodiffusion plates (ICL Scientific, Fountain Valley, California, U. S. A.) by the kinetic method. Aliquots of the patients' serum (5 µl) were placed in each well and the diameter of the precipitin ring was measured 18-24 hours

Table 1. The dilutions of antigen used and the criteria for positive reactions of the delayed hypersensitivity skin tests

Antigen	Stock concentration	Dilution used	Criterion for positive reaction
Tuberculin P.P.D. B.P. (Evans Medical, Liverpool)	100000 u/ml	1:2000	Induration, 1 cm or greater
Candida (Monilia) Albicans (Hollister-Stier Labs., Spokane, U. S. A.)	1:10 w/v	1:1000	Induration, 1 cm or greater
Trichophytin (Dermatophytin) (Hollister-Stier Labs., Spokane, U. S. A.)	Undiluted	1:20	Induration, 1 cm or greater
Streptokinase/Streptodornase (Varidase) (Lederle Labs., Hants)	Streptokinase 100000 units Streptodornase > 25000 units	1:20	Induration, 2 cm or greater

later. By comparing the diameter of these rings with those produced by known concentrations the amount of each immunoglobulin was determined. The results were compared with a series of measurements from normal individuals in whom concentrations of immunoglobulins varied: IgG from 5-16, IgA 1.25-4.25 and IgM, 0.5-2.0 g/100 ml (Tee, personal communication).

RESULTS

(1) Makari Skin Test. Positive Makari skin tests were observed in 15/21 (71%) of the Group 1 (recurrent tumour) patients, compared with 6/22 (27%) of those in Group 2 (disease-free).

(2) Cell-Mediated Immunological Competence. The results from the three groups of patients are compared in Table 2. No significant difference was observed between the two bladder cancer groups in the lymphoproliferative response to PHA with either pooled AB or autologous serum (see columns 3 and 4 in Table 2). However, both groups showed a significantly reduced response in comparison with the control group (p values: no recurrence/AB serum < 0.01, autologous serum < 0.05; with recurrent tumour/AB serum < 0.01, autologous serum < 0.001). These results were not altered by exclusion of the patients previously treated with radiotherapy or chemotherapy.

In 6/22 of the Group 2 and in 5/21 Group 1 patients there was a decrease of more than 25% in the T/C ratio in the presence of autologous as compared with pooled AB serum, suggesting that in these cases serum factors were depressing the immune response. However, a similar decrease was observed in two of the control group.

The lymphoproliferative response to PHA (T/C ratio) was significantly higher in patients with positive delayed hypersensitivity skin reactions than in those with negative reactions ($p<0.05$) in the presence of autologous serum. An association between these two measures of cell-mediated immunocompetence could be expected. However, the numbers of T and peripheral blood lymphocytes in the two bladder cancer groups did not differ significantly from each other, nor did these estimates differ significantly from the counts in the control group (see columns 5 and 6 in Table 2).

(3) Serum Immunoglobulins. There was no significant difference in the serum levels of the three immunoglobulins between the two bladder cancer groups (see Table 3). In addition, when the serum levels were compared with those of normal individuals no clear-cut differences were observed between the two groups, although raised IgG levels were more common in the disease-free group.

(4) Correlation of the Makari Skin Test with Measurements of Immunocompetence. Of the 43 bladder cancer patients 22 had negative and 21 positive Makari skin tests. None of the tests of

Table 2. Comparison of the measurements of cell-mediated immunocompetence between the two bladder cancer and control groups. SD = Standard Deviation

Patients	Makari skin test positive	Positive response to one or more 'recall' antigens (delayed hypersensitivity skin reaction)	Response to PHA T/C ratio ± S.D. (range)		T cell numbers ± S.D.	Number of blood lymphocytes ± S.D.
			AB serum	autologous Serum		
Group 1 with recurrent tumour (21 patients)	15 (71%)	6/15 (40%)	16.5 ± 19.9 (1.9-87.3)	12.9 ± 10.8 (2.5-37.2)	1295 ± 518	2209 ± 920
Group 2 clinically disease-free (22 patients)	6 (27%)	4/12 (33%)	16.7 ± 18.1 (2.1-64.7)	17.0 ± 22.0 (1.8-81.4)	1279 ± 593	2133 ± 978
Group 3 benign prostatic hyperplasia (20 patients)	not done	not done	32.3 ± 15.9 (9.0-78.0)	30.2 ± 12.0 (12.0-65.0)	1507 ± 690	2261 ± 795

Table 3. Combined data comparing the levels of serum immunoglobulins and the numbers of cases in which the serum levels were beyond the normal range between the two bladder cancer groups. SD = Standard Deviation

Patients	Serum immunoglobulins g/100 ml ±S.D.			Number of patients with serum immunoglobulin levels outside the normal range					
				IgG(a)		IgA(b)		IgM(c)	
	IgG	IgA	IgM	raised	lowered	raised	lowered	raised	lowered
Group 1 with recurrent tumour (21 patients)	12.4 ± 2.9	2.2 ± 0.8	1.2 ± 1.2	1 (5%)	0	0	3 (14%)	7 (33%)	0
Group 2 clinically disease-free (22 patients)	13.5 ± 3.9	2.4 ± 1.0	1.7 ± 1.0	4 (18%)	0	1 (5%)	3 (14%)	7 (32%)	1 (5%)

(a) IgG normal range 5-16 g/100 ml
(b) IgA normal range 1.25-4.25 g/100 ml
(c) IgM normal range 0.5-2.0 g/100 ml

Table 4. Comparison of the measurements of cell-mediated immunocompetence between the Makari negative and positive patients. S.D. = Standard Deviation

Patients	Positive response to one or more 'recall' antigens (delayed hypersensitivity skin reaction)	Response to PHA T/C ratio ± S.D. (range) AB serum	autologous serum	T cell numbers ± S.D.	Number of peripheral blood lymphocytes ± S.D.
Makari skin-test negative (22 patients)	6/14 (43%)	14.9±17.0 (2.1-64.7)	16.4±21.6 (1.8-81.4)	1266 ±615	2119 ± 1005
Makari skin-test positive (21 patients)	4/13 (31%)	18.5±20.7 (1.9-87.3)	13.5±11.7 (2.3-36.5)	1308 ± 490	2224 ± 888

Table 5. Comparison of the levels of serum immunoglobulins and the number of cases in which serum levels were beyond the normal range between Makari negative and positive patients. S.D. = Standard Deviation

Patients	Serum Immunoglobulins g/100 ml ± S.D.			Number of patients with serum immunoglobulin levels outside the normal range					
				IgG(a)		IgA(b)		IgM(c)	
	IgG	IgA	IgM	raised	lowered	raised	lowered	raised	lowered
Makari skin-test negative (22 patients)	12.8±3.2	2.3±0.8	1.8± 1.2	2 (9%)	0	0	2 (9%)	7 (32%)	1 (5%)
Makari skin-test positive (21 patients)	13.2±3.8	2.3±1.0	1.9±1.0	3 (14%)	0	1 (5%)	4 (19%)	7 (33%)	0

(a) IgG normal range 5-16 g/100 ml
(b) IgA normal range 1.25-4.25 g/100 ml
(c) IgM normal range 0.5-2.0 g/100 ml

cell-mediated immunocompetence used in this investigation showed a significant difference between Makari positive and negative patients (see Table 4). Similarly, no differences were observed in the levels of serum immunoglobulins (see Table 5).

DISCUSSION

The purpose of this study was to investigate a possible correlation between the Makari skin test and cell-mediated and humoral immunocompetence. In the present study 15/21 (71%) of patients with recurrent disease (Group 1) had positive Makari skin tests. Previous studies have shown that by excluding 'anergic' patients (those with a negative delayed hypersensitivity skin reactions) most of the false negatives are excluded (4, 15, 23). In this series six were classed as false negatives; three were anergic, two had positive delayed hypersensitivity skin reactions, and one was not tested for delayed hypersensitivity. Excluding the 'anergic' patients and the patient who was not tested the Makari skin test detected malignant disease in 15/17 (88%) of the cases, confirming its value as a test for the detection of recurrent bladder cancer. A clinical test for malignancy must also result in a low percentage of false positives. In this series there were 6/22 (27%), but previous studies have shown that the Makari skin test can detect bladder cancer before it is clinically apparent (15, 16, 23). Therefore a longer follow-up than has been so far possible in these cases will be necessary to determine the true incidence of false positives.

Impaired cell-mediated immunocompetence has been demonstrated in association with many types of neoplasia. A number of studies of delayed hypersensitivity skin tests in bladder cancer patients have been made using a variety of 'recall' antigens (8, 14, 17, 20), and previously unencountered antigens such as 2,4 dinitrochlorobenzene (1, 8, 20), 2, 4 dinitrofluorobenzene (6), croton oil (8) and keyhole-limpet hemocyanin (17). Direct comparison of these various tests is not valid due to differences in methodology, types of patient and treatment, but it does appear that the proportion of bladder cancer patients able to produce a delayed hypersensitivity skin reaction is reduced compared with controls. In the present study only 10/27 (37%) of patients showed a positive response to one or more 'recall' antigens, and these results are not significantly different from previous findings.

The lymphoproliferative response to PHA was also reduced in the two groups of bladder cancer patients in this study. In addition, the lymphoproliferative response to PHA of the patients with negative delayed hypersensitivity skin reactions was significantly lower than those with positive reactions, in the presence of autologous serum, demonstrating the association between these two measurements of cell-mediated immunocompetence. Previous studies on the lymphoproliferative response to mitogens have also shown a reduction in response in bladder cancer patients (2, 7, 10, 14). However, the published results are conflicting with regard to the relationship between reduction in lymphoproliferative response and pathological stage. In the present study all the tumours were pathological stage P1 or P2 and the response was reduced compared with that of the control group whether or not recurrent disease was clinically evident. This correlation was maintained when the patients who had been irradiated or treated with chemotherapy were excluded, confirming the finding of McLaughlin (10) that cell-mediated immune competence can be reduced even in the early stages of bladder cancer.

In contrast to the other measurements of cell-mediated immunocompetence there was no reduction in the numbers of circulating T-lymphocytes or peripheral blood lymphocytes in either bladder cancer group, in comparison with the control group. This observation is in agreement with Elhilali (7), who found no significant reduction in the numbers of T-lymphocytes in patients with clinically localised cancer, although this data are derived from a mixed group of bladder, prostate and kidney cancer patients. In contrast, Catalona et al. (3) found a significant reduction in the numbers of T-lymphocytes in 21 bladder cancer patients, and the reduction was significant in both local and invasive cancer.

With regard to humoral immunocompetence, no major difference was observed in the levels of serum immunoglobulins between those patients with and without recurrent disease. However, measurement of serum immunoglobulins is a relatively inexact method of determining humoral immunocompetence, and more refined methods, such as measurement of the numbers of B cells, might have revealed differences between the two groups. In one-third of the patients in each group the levels of IgM were raised. In a study of 10 bladder cancer patients Merrin and Han (14) found that immungoglobulin levels were normal in all the patients studied.

No correlations were found in this small series between the Makari skin test and measurements of immunocompetence, confirming an earlier study on breast cancer patients (Tee, unpublished result). However, the Makari skin test does appear to be associated with immunological competence, because exclusion of 'anergic' patients results in a greater accuracy of detection of carcinoma (4, 15, 16, 23). In addition, a correlation was observed (5) when the test was compared with the total number of immunological deficiences in 37 patients with lung cancer (de-

termined by measuring lymphocyte stimulation in vitro with PHA, Concanavalin A and pokeweed mitogen, T and B cell numbers, peripheral blood lymphocyte levels, and delayed hypersensitivity skin reactions to five microbial antigens and de novo sensitization with dinitrochlorobenzene). However, the only individual measurement of immunocompetence which was correlated with the Makari skin test was the delayed hypersensitivity skin reaction. Thus, a definitive characterisation of the Makari skin test has yet to be made.

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