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THE CELLULAR IMMUNE RESPONSE TO CARCINOMA OF THE URINARY BLADDER: CORRELATION TO CLINICAL STAGE AND TREATMENT

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Summary.—The cell mediated immune response to carcinoma of the urinary bladder in man is influenced significantly by the tumour burden before treatment. Therapy appears to influence this response by causing alterations in the amount of tumour material in the body. Removal of tumour by surgery is seen to result in a loss of detectable CMI. Recurrence of tumour after surgery results in the reappearance of cytotoxicity. Treatment by radiotherapy also results in the eventual loss of CMI after the elimination of tumour material from the body. The loss of activity after radiotherapy, in the absence of tumour recurrence, occurs over a period of about 1 year. Expression of CMI is suppressed during radiotherapy but may return after treatment. Failure to develop lymphocyte cytotoxicity early after radiotherapy is related to the presence of metastases or residual tumour. Low levels of cytotoxicity during the first 9 months after therapy are associated with tumour recurrence. It may be inferred from this that CMI in the early post-irradiation phase has prognostic significance. The absence of CMI at this time reflects the presence of residual viable tumour in the body. The loss of response about 1 year after radiotherapy probably reflects the clearance of tumour-derived material from the body. The persistence or reappearance of cytotoxicity after this time is related to tumour recurrence.

This test is therefore informative as to the presence or absence of tumour after surgery. With regard to radiotherapy, lymphocyte cytotoxicity can be seen to monitor the presence of viable tumour and tumour derived material in the body.

DISTINCTIVE cellular and humoral immune responses have been described to a range of human and animal neoplasms (Hellström and Hellström, 1969; Morton Eilber and Malmgren, 1971; Bubeník *et al.*, 1970*a*, *b*). The existence of a common antigenicity among tumours of the same histogenic origin has permitted the use of allogeneic test systems for tumour immunity. In the study of cell mediated immunity to tumour (CMI) in patients with transitional carcinoma of the urinary bladder (TCC) it has been observed that lymphocyte cytotoxicity can be measured on both primary cultures and cell lines derived from the relevant tumour type. The use of cell lines derived from malignant and non-malignant material of relevant histogenic origin has facilitated standardization of the test system (O'Toole *et al.*, 1972*a*, *b*). It has thus been possible to obtain a quantitative and reproducible assay of CMI (O'Toole, 1973).

Quantitative changes in the cellular and humoral immune responses to tumour have been observed with tumour progression (Barski and Youn, 1969; Lewis *et al.*, 1969; Morton *et al.*, 1971). In patients with TCC the incidence of CMI has been seen to vary with tumour burden (O'Toole *et al.*, 1972*a*, *b*). CMI is seen to reflect the relative quantity of tumour material in the body both before and after therapy.

MATERIAL AND METHODS

Clinical data.—Carcinoma of the urinary bladder (TCC) was staged according to the U.I.C.C. system (1963) and graded histo-

logically by the system of Bergkvist, Ljungqvist and Moberger (1965).

Radiotherapy was given in local tumour doses of 6500–7000 rad over a 7–8 week period. Alternatively, 2 courses of 4200 rad separated by a 2 week interval were given. Patients given preoperative radiotherapy received 3500–4000 rad over a 4–6 week period, followed by total cystectomy after a 4–6 week interval. Further details of the irradiation techniques and schedules have been published elsewhere (O'Toole *et al.*, 1972*a*, *b*).

Surgery was performed by total cystectomy or transurethral resection.

Sequential tests were performed on patients with TCC in the following situations: before treatment, during and after radiotherapy and after surgery. Individual patients have been studied during a 2-year follow-up period after treatment. Patients with other types of malignancy, benign tumours or infections of the urinary tract were tested as clinical controls; these are summarized in Table I. Where possible patients with TCC and clinical controls were tested in similar clinical situations. Healthy individuals were also compared with the clinical groups.

Cell mediated immunity was assayed as described previously (O'Toole, 1973). Specific CMI could only be quantitated if highly purified lymphocyte preparations were used.

Lymphocytes were prepared routinely as follows. About 30 ml of defibrinated blood was obtained from each donor for each test. Erythrocytes were sedimented through gelatin as described by Coulson and Chalmers (1964) and then adherent cells were removed by incubation of the resulting leucocyte preparation on a nylon wool column by the method of Greenwalt, Gajewski and McKenna (1962). Residual erythrocytes were then lysed by the tris-ammonium chloride method (Boyle, 1968). The cells were washed 3 times with tris-Hank's solution containing 2·5% heat inactivated foetal calf serum, after each of the steps described. The lymphocyte preparations obtained were usually of $\geqslant 95\%$ purity; this was confirmed on all preparations stained with May–Grünwald Giemsa.

Target cells.—Three cell lines derived from human bladder formed the basic test material. These were RT4 (Rigby and Franks, 1970) and T24, derived from TCC and HCV/29 (supplied by Dr J. Føgh, Sloan Kettering Institute, New York) an epithelioid line of non-tumour origin. Primary cultures of TCC were also tested in parallel with the cell lines. Cells of non-bladder origin have also been tested; the control target cells are summarized in Table I.

The microcytotoxicity assay for CMI was based on that described by Takasugi and Klein (1970). Target cells prepared by trypsinization from monolayer cultures were seeded at a concentration of 20–50/well on to microplates (Falcon 3034). The culture medium used routinely was 199 with 10% foetal calf serum (heat inactivated 56°C, 60 min), containing 100 i.u. penicillin, 100 μg streptomycin and 0·3 mg glutamine/ml. The plates were then incubated for 3–12 hours at 37°C in humidified air + 5% CO_2 to permit cell attachment. After this time lymphocyte preparations at a concentration of 1×10^6 cell/ml and 1 or 2 doubling dilutions thereof were added to the target cells. Twelve or 18 wells of each target cell type under test were used for each donor's lymphocytes at a given concentration. On each plate a further 12 or 24 wells were incubated with medium only to control target cell

TABLE I.—*Specificity Controls for CMI to Carcinoma of the Bladder*

Lymphocyte donors diagnosis		Target cells	
Normal healthy	30	*Cell lines*	Origin
Chronic cystitis	10	HCV/29	Normal bladder epithelium
Urethritis	4	2T	Osteogenic sarcoma
Ca prostate*	9	Chang	Liver
Other tumours of the urogenital tract	7	*Primary cultures*	
Ca mammae†	4	Renal ca	Epithelial
Ca metastatic to bladder‡	4	Lung	Fibroblasts
		Bladder	Fibroblasts

* Lymphocyte samples obtained from 7 donors before, from 2 of these during, and from 3 after, radiotherapy.

† Lymphocytes obtained from 2 donors before, and from 3 during radiotherapy.

‡ Primary tumour in colon, 2 cases, in stomach, 1 case, in rectum, 1 case.

viability. After incubation for 24–40 hours, the plates were washed with phosphate buffered saline, stained with May–Grünwald Giemsa and the remaining target cells estimated visually.

Evaluation of CMI.—The average number of residual target cells in wells which had contained lymphocytes from patients with carcinoma of the bladder was compared with that in wells incubated with control donors' lymphocytes, at equivalent concentrations. The differences were compared by Student's *t*-test with a significance level of $\leqslant 0{\cdot}05$. Cytotoxicity is expressed as the % reduction in target cell number produced by lymphocytes from patients with TCC compared with control donors' lymphocytes (= 100).

RESULTS

The specificity of the cell mediated immune response to TCC has been described in detail elsewhere (Bubenik *et al.*, 1970*a*, *b*; O'Toole *et al.*, 1972*a*, *b*). Cell lines derived from bladder epithelium can be used as reference target cells, as shown in Table II. The lines T24 and RT4 derived from TCC are destroyed by lymphocytes from patients with TCC; HCV/29 derived from bladder epithelium is not specifically affected. The microcytotoxicity test under the conditions described has been found to give a quantitative and reproducible assay of CMI (O'Toole, 1973). It has thus been possible to study the effect of the following parameters.

Effect of tumour staging on the incidence of CMI.—Before therapy 88% of patients with small localized tumours stages T1 or T2 were found to have lymphocytes specifically cytotoxic for TCC cells. Significantly, only 41% of patients with larger tumours, stage T3 or T4, had such a response. The malignancy grade of the tumour did not appear to have as great an influence as staging on the incidence of CMI, but in general these 2 parameters correlated, the more malignant tumours having higher staging.

Effect of treatment on CMI

Radiotherapy.—Local tumour therapy in doses greater than 1000 rad resulted in the loss of detectable lymphocyte cytotoxicity. However, after radiotherapy in total doses of 4000, 6500 or 8400 rad cytotoxicity was detected in some patients. As shown in Fig. 1, this response could occur after a 3-day lag period. In patients receiving 2 courses of radiotherapy separated by a 2-week interval, the second course of therapy also gave a suppression of CMI in doses above 1000 rad (Fig. 2).

Following radiotherapy, it was observed that all patients with low stage tumours T2 redeveloped cytotoxicity, as illustrated in Fig. 1. However, some of the patients with higher stage tumour failed to redevelop their pre-irradiation responses (Fig. 3). Alternatively, some patients who had no CMI before treatment developed a response after radiotherapy (Fig. 2). These observations have been published in detail elsewhere (O'Toole *et al.*, 1972*a*, *b*) and are summarized in

TABLE II.—*Specificity of the Cytotoxic Reaction on Cell Lines Derived from Human Bladder*

Cell lines	E : T[1]	Control[2] lymphocytes	Patient's[3] lymphocytes	% reduction[4]
HCV/29*	500 : 1	55±15	52±9	*4*‡
	250 : 1	73±12	76±9	*0*
T24†	500 : 1	56±13	20±10	*64*
	250 : 1	56±8	35±9	*37*
RT4†	500 : 1	63±15	35±10	*44*
	250 : 1	60±16	51±14	*15*

* Normal bladder origin. †Ca bladder. ‡ Not significant.
[1] Effector : target cell ratio.
[2] Healthy donor.
[3] Ca bladder T2. M2 untreated.
[4] Estimated on control donor's lymphocytes (= 100). For details see Material and Methods.

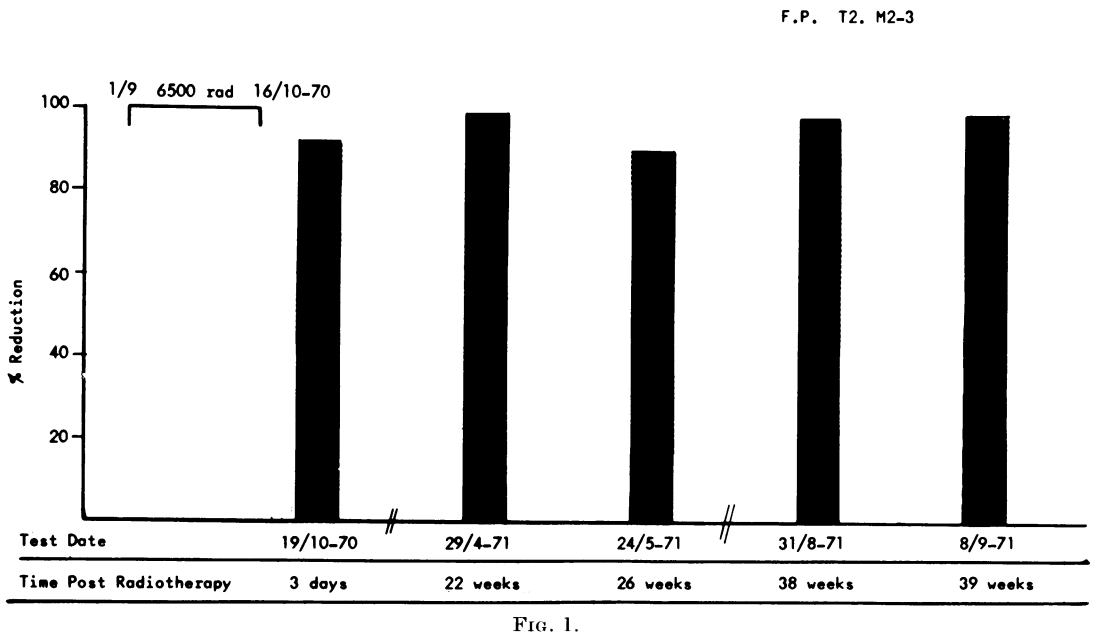
F.P. T2. M2-3
1/9 6500 rad 16/10-70
100
80
60
40
20
% Reduction
Test Date
19/10-70
29/4-71
24/5-71
31/8-71
8/9-71
Time Post Radiotherapy
3 days
22 weeks
26 weeks
38 weeks
39 weeks

FIG. 1.

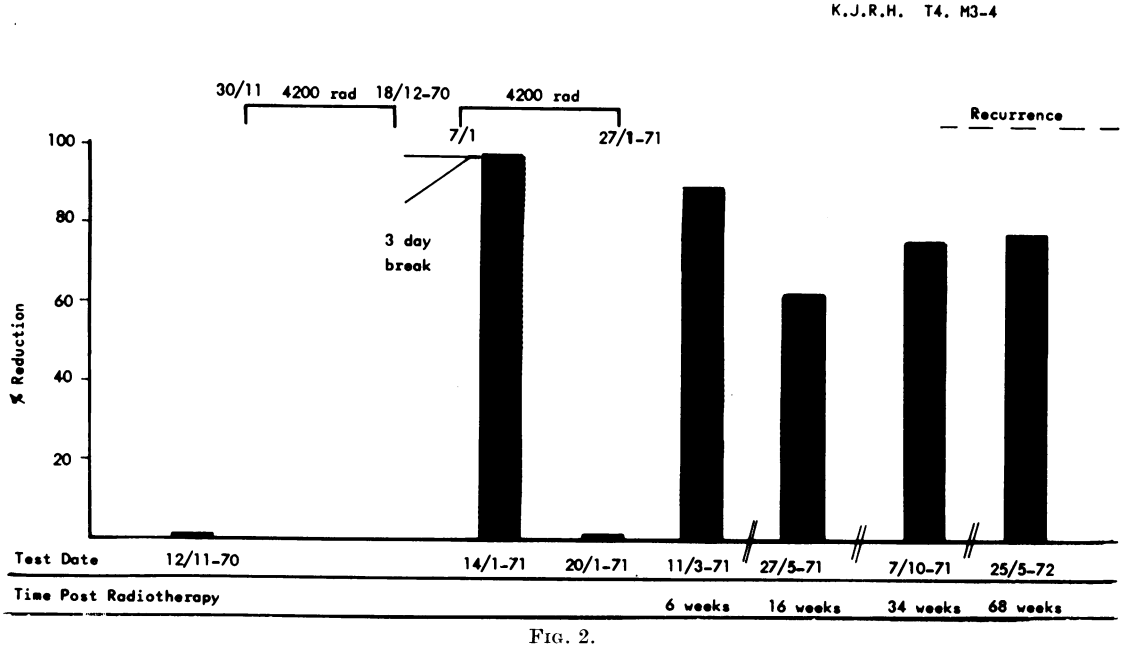
K.J.R.H. T4. M3-4
30/11 4200 rad 18/12-70
4200 rad
7/1
27/1-71
Recurrence
3 day
break
100
80
60
40
20
% Reduction
Test Date
12/11-70
14/1-71
20/1-71
11/3-71
27/5-71
7/10-71
25/5-72
Time Post Radiotherapy
6 weeks
16 weeks
34 weeks
68 weeks

FIG. 2.

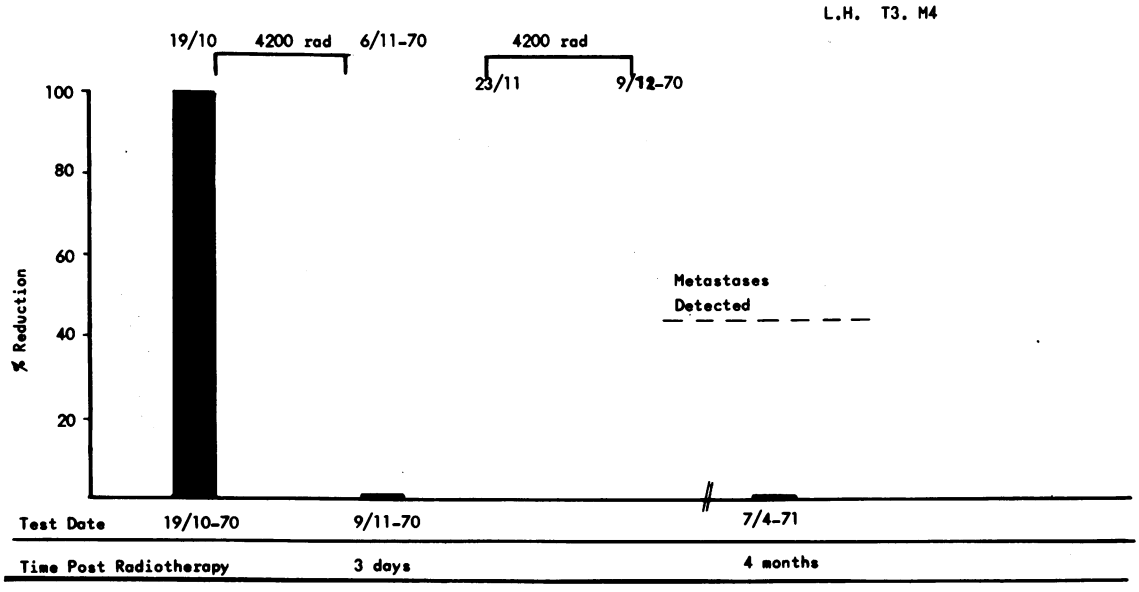

FIG. 3.

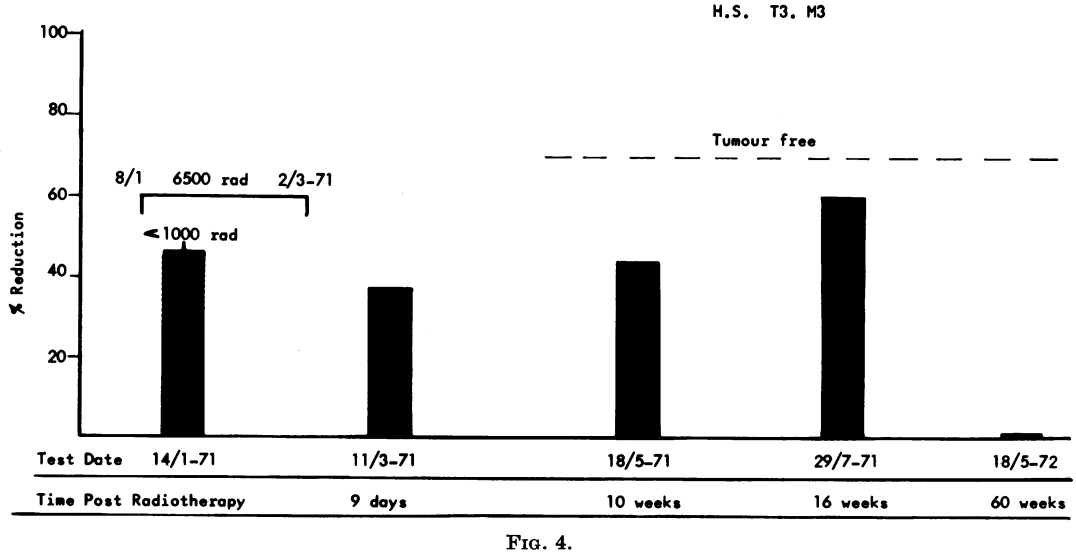

FIG. 4.

FIG. 1–4.—TCC patients treated by radiotherapy, 6500–8400 rad. % reduction estimated as described in Material and Methods. These results were obtained with a lymphocyte : target cell ratio of 500 : 1. T24 and RT4 were used as reference target cells. No specific effects were observed on control target cells.

TABLE III.—*Incidence of CMI in Patients given Radiotherapy*

		Tumour stage		
		T2	T3	T4
Untreated		14/16	4/9	3/8
During treatment				
Doses > 1000 rad		0/3	0/4	0/6
Post radiotherapy				
≤3 months		5/5	8/10*	4/8*
3–9 months	Strong CMI, no tumour	2/2	5/5	2/2
3–9 months	Weak CMI, tumour	3/3†	2/2†	1/1†
≥1 year	No tumour	0/2	0/4	—
≥1 year	Tumour	—	2/2	2/2
2–12 years	No tumour	0/3	0/3	0/4
2–12 years	Tumour	1/2	3/3	2/2

* Negative patients had either metastases or large residual tumours during this period.
† Patients developed recurrences or metastases during this period.

TABLE IV.—*Incidence of CMI in Patients Treated by Surgery*

	Tumour stage		
	T1/T2	T3	T4
Untreated	12/13	—	—
After resection			
No tumour	0/10	0/1	—
Recurrence	4/4	—	—
After total cystectomy			
No tumour	0/4	—	—
Residual tumour	1/1	—	—
After preoperative radiotherapy and cystectomy			
< 1 year, no tumour	3/4*	—	1/1
> 1 year, no tumour	0/2	—	0/1
> 1 year, recurrent tumour	1/1	—	—

* Negative patient developed metastases. 9 months after surgery.

Table III. Retrospectively, it was seen that those patients who lacked cytotoxicity during the first 3 months after radiotherapy had either large residual tumours and/or metastases during this time; this course of events is typified by patient L.H. (Fig. 3). During the first 9 months post radiotherapy, patients with strong cytotoxicity reactions (giving ⩾ 50% reduction in tumour cell survival) showed no clinical evidence of tumour. Lower levels of cytotoxicity were associated with tumour recurrence or metastases (Table III). In the absence of tumour recurrence the post-irradiation CMI was seen to disappear after about 1 year (Fig. 4). Patients who had high levels of cytotoxicity during the first year post therapy, but whose responses failed to decline after this time, were found to have tumour recurrences (Fig. 2 and Table III).

Long-term observations post irradiation.—A group of patients treated 2–12 years previously were also tested for CMI to tumour. The pattern observed was identical to that of patients followed more than 1 year after therapy. The presence of specific cytotoxicity was associated with the presence of tumour in the body (Table III).

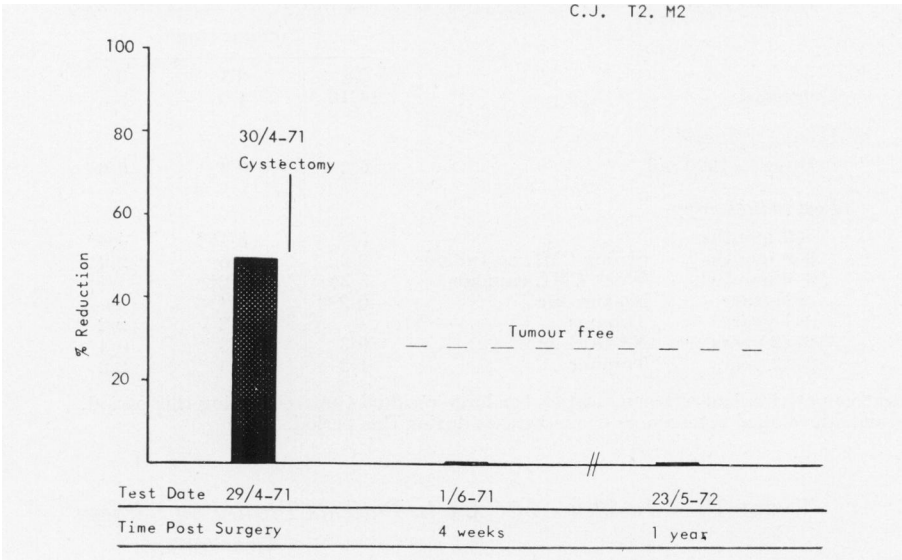

FIG. 5.

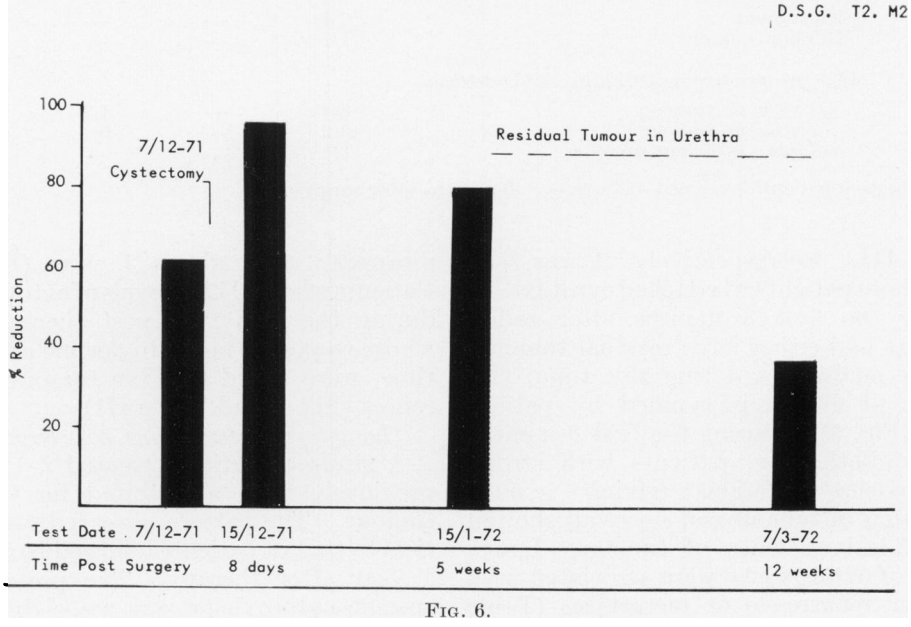

FIG. 6.

FIG. 5–6.—TCC patient treated by total cystectomy. % reduction see Fig. 1.

Surgery.—Patients treated by surgery only, either local resection or total cystectomy, had in general small localized tumours and so a high incidence of CMI before treatment. Removal of clinically detectable tumour in these patients was followed by the loss of existing cytotoxicity during a 4-week observation period (Fig. 5). However, in 1 patient (Fig. 6) cystectomy resulted in an increase in CMI. This patient differed from the other surgical cases studied in that although the staging of the tumour was T2, a large tumour was present which filled the lumen of the bladder. In addition this patient was found to have residual tumour foci in the urethra after cystectomy. Reappearance of cytotoxicity in patients treated by surgery was always associated with tumour recurrence. The data are summarized in Table IV; for details see O'Toole *et al.* (1972*a*, *b*).

Preoperative radiotherapy.—The patients in this group showed similar post-therapy patterns of CMI to those receiving radiotherapy only (Fig. 7). Total cystectomy 2 weeks after radiotherapy did not appear to influence existing post-radiotherapy cytotoxicity.

DISCUSSION

The maintenance of cellular immunity to TCC is clearly dependent on the presence of critical amounts of tumour material in the body. This may be inferred from the loss of response in tumour-free patients and its reappearance with tumour recurrence. The lower incidence of cytotoxicity in untreated patients with high stage tumours suggests that large amounts of tumour material can inhibit this response. Inhibition of both cellular and humoral immune responses to tumour by increasing tumour burden have been described (Barski and Youn, 1969; Lewis *et al.*, 1969; Morton *et al.*, 1971).

Radiotherapy appears to have a paradoxical role exerting both suppressive and stimulatory effects on this response. As previously described (Ilbery, Rickinson and Thrum, 1971; Stjernswärd *et al.*, 1972), local radiotherapy can result in

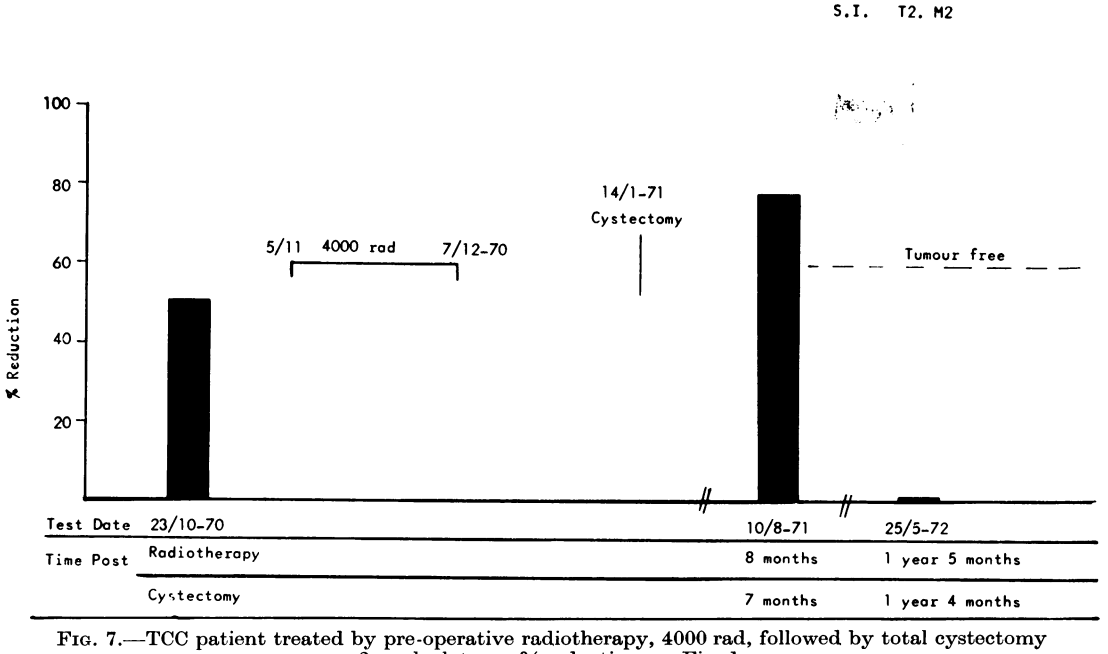

FIG. 7.—TCC patient treated by pre-operative radiotherapy, 4000 rad, followed by total cystectomy 2 weeks later. % reduction see Fig. 1.

peripheral lymphopenia, which persists for several months after therapy. This suggests that the patient in the early post-radiotherapy phase can have a reduced immunocompetence. In this situation a lack of response to remaining viable tumour cells would be expected to result. The early appearance of metastases in patients lacking CMI during this time could be explained on this basis. Similarly patients with a weak cytotoxic response during the first 9 months after therapy showed early evidence of recurrence or metastases during this period.

The immune response detected in this system is radiosensitive; doses about 1000 rad were seen to suppress both pre-existing responses and also those developing after an earlier course of radiotherapy. The rapid recovery of CMI observed in some patients after radiotherapy is noteworthy; responses could be detected within 3 days in some cases. Analogous results have been reported after chemotherapy, where a recovery of the immune response to a variety of antigens was observed (Harris and Stewart, 1972). Development of lymphocyte cytotoxicity to tumour post radiotherapy is likely to be dependent on the quantity of tumour material in the body at this time also. The induction of specific CMI to tumour after therapy in some patients who previously lacked this suggests that, depending on the quantity of material released during tumour necrosis, either stimulation or inhibition of CMI may result.

The maintenance of cytotoxicity after surgery in patients given pre-operative radiotherapy indicates that radiotherapy causes the peripheralization of tumour derived material. The duration of this response was similar to that in patients given radiotherapy only, although the pre-operative group received lower doses of radiotherapy. The loss of reactivity in tumour-free patients about 1 year after radiotherapy suggests that the stimulatory material is gradually cleared from the body.

Development of CMI to this tumour would seem therefore to depend on a fine balance between quantity of tumour material in the body and the functional capability of the patients' immune system.

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