Urol Res (1985) 13:123–130

Urological Research

Quantitative Measurements of Humoral Immune Response in Mice to a FANFT* Induced Bladder Tumor**

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Accepted: February 27, 1985

Summary. Using unfixed mouse bladder tumor cells (MBT) as target cells and a modified avidin-biotin-complex (ABC) method made it possible to detect a humoral immune response in C3H mice with growing MBT tumors. The rise of the serum levels is significant ($p < 0.005$) when compared to control animals and correlates with the tumor size. Mice with recurrences after surgical removal of the primary tumor had significantly ($p < 0.05$) higher serum values than animals without recurrence. Purulent or granulomatous inflammatory changes, muscle necrosis, growth of a lymphoma or an ovarian carcinoma did not significantly change the results ($p < 0.05$). Injection of myeloma cells from a different strain of mice caused a minimal, but significant increase in the values when compared to controls ($p < 0.0001$). However, the slope of the curve differed significantly ($p < 0.05$) from that in mice with MBT tumors. Irradiated MBT cells or *Corynebacterium parvum* used as immunomodulators significantly increased the serum values ($p < 0.001$). In the presence of a growing MBT tumor, however, the immunomodulation did not substantially falsify the results. The serum values were related to the corresponding tumor sizes.

Key words: Humoral immune response, MBT-2 tumor, Serum marker, Immunomodulators, Monitoring of tumor growth, Avidin Biotin Complex

Only a few malignant neoplasms can be detected by serum markers. Amongst these oncofetal proteins [e.g., alpha-fetoprotein (AFP) carcinoembryonic antigen (CEA)], and other markers such as the Bence Jones protein in multiple myeloma have been described. For most carcinomas, however, which do not produce specific pathognomonic markers, indirect methods based on immunological characteristics provide valuable clinical tests. Circulating tumor-associated antigens (TAA) shed by the tumor [2, 6] as well as antibodies against various malignant human tumors, e.g., renal cell carcinoma [1], colon carcinoma [10], melanoma [9], uterine cervix carcinoma [15] or transitional cell carcinoma [3, 7, 14] have been reported, as well as antigen-antibody complexes [11]. However, the sensitivity and specificity of the procedures used, such as the antibody-dependent lymphocyte mediated cytotoxicity test (ADCC) or immuno-histochemical procedures [immunofluorescence, the peroxidase-anti-peroxidase method (PAP) or radioimmunoassays (RIA)] were either unsatisfactory or the technique too complicated to be used routinely for the detection or follow-up of most human cancers.

This study was undertaken to explore the reliability of a binding assay as a measure of humoral immune response. The avidin-biotin-complex (ABC) method developed by Hsu and coworkers was used [8]. When sera from patients with renal cell carcinoma were exposed to unfixed cells of a human renal cell cancer tissue culture cell line (RC-Pa), we measured a significantly higher peroxidase activity compared to sera from patients with other cancers or to sera from healthy donors (J Urol, in press).

Moreover, preliminary work indicates that a similar technique (with the cell line J-82 as antigen) could likewise be used in patients with bladder cancer. In this study, an animal model was used to study the influence of factors such as size and location of the tumor and immunomodulation and infections on this modified ABC method, thus to obtain more information about its potential usefulness as a serum marker assay.

Materials and Methods

Tumor Cells. Mouse bladder tumor cells (MBT-2) originally induced in C3H/HeN mice by chronic feeding of N-4-(5-nitro-2-furyl)-2 thia-

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* N-4-(5-nitro-2-furyl)-2 thiazol formamide

** Supported by the Swiss Foundation for Biological and Medical Research and the Swiss Cancer League, The Blalock Foundation, and the USPHS Grant CA-16042

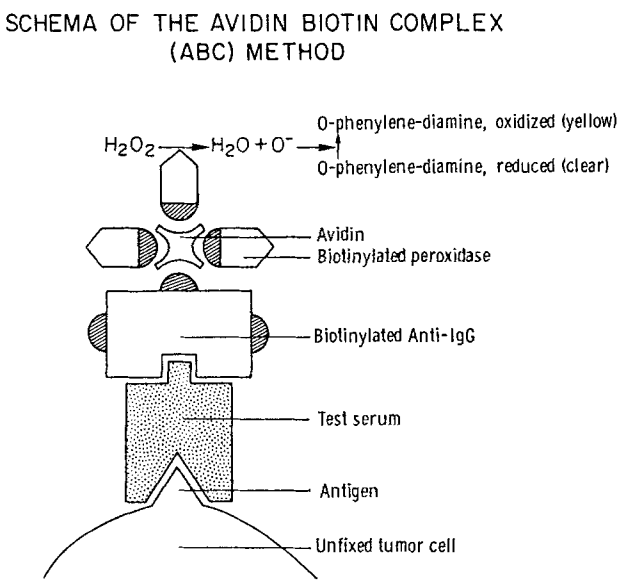


Fig. 1. Schema of the modified avidin-biotin-complex (ABC) method

zolyl formamide (FANFT) were provided by M. Soloway, Memphis, Tennessee. Between passages in C3H/NeH mice, they were maintained as a stationary culture in RPMI-1640 medium (Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum. The lymphoma cells 6C3HED and the non-secreting myeloma cell line S194/5.XXO.BUI were similarly passed in vitro, the latter derived originally from Balb-C mice [13]. The tumor cell suspensions were harvested by trypsinization (0.25%) for approximately 1 min, mechanically dispersed (by pipette) and washed twice with 0.1 M phosphate-buffered saline, pH 7.1 (PBS). The ovary teratocarcinoma cells (MOT) [5] were passed in vivo (intraperitoneally, ip) in C3H/HeBFeJ mice. The concentration per ml of the tumor cell suspensions was 10 times higher than the number of cells that had to be injected, thus allowing injection of 1/10 ml of the cell suspension. Viability of the cells was assayed by the trypan blue exclusion method.

Various Methods. Control animals were injected with 0.1 ml PBS buffer in RHL unless otherwise specified. In order to provoke histologically proven granulomatous inflammatory reactions, 0.1 ml of mineral oil was used. Chronic abscesses were produced by 1 mg of heat-killed *Aspergillus fumigatus* mycelium, strain 13,073 [4]. These agents were injected into the thigh of the right hind leg (RHL). Muscle necrosis, hematomas and scarring were provoked by daily mechanical injury to the RHL.

Immune Response Modifiers. MBT cells (10^7) irradiated with 15,000 rads immediately prior to injection, 700 μg of *Corynebacterium parvum* (C. Parvum), 700 μg of lyophilized *Bacillus Calmette Guerin* (BCG) type Pasteur, Paris, or combinations thereof, were injected alone or simultaneously with the injection of living MBT cells in the RHL. The intraperitoneal injection of the immunomodulators was repeated once 3 days later.

Animals. For the first 2 experiments 6- to 8-week-old C3Hf/Sed mice obtained from Sedlacek, Boston, were used [12]. In the following experiments, 6- to 8-week-old C3H/NeNCr1BR female mice were obtained from Charles River Laboratory, Massachusetts. The female mice injected intraperitoneally with MOT were 6 months old and of the subspecies C3HeB/FeJ.

Serum Sampling. Three mice from each group were randomly chosen to be sacrified, bled and their sera pooled and frozen at −70 °C. At that time the maximal diameter of the tumors was measured with a caliper.

Immunoassay. Monolayers of MBT cells were harvested and dispersed as described above. 2×10^7 unfixed cells were aliquoted into siliconized test tubes and washed twice with 0.05 M TRIS buffer (pH 7.6, supplemented by 0.05 ml/TWEEN per liter). Washing was repeated between each of the following steps:

0.4 ml of normal diluted horse serum (blocking serum, 45 μl/2.5 ml Tris buffer).

0.4 ml of 1:100 diluted test serum,

0.4 ml of biotinylated horse anti-mouse IgG (45 μl/10 ml TRIS-buffer),

0.4 ml of diluted "Vectastain" (90 μl of avidin and 90 μl of biotinylated horseradish peroxidase in 10 ml TRIS buffer).

The cells were inucubated with each of the reagents (Vector Laboratories, Burlingame, CA 94010, USA) for 30 min at 37 °C. At each of the repeated intermediate cell-washings, the cells were resuspended (Vortex) in approximately 4 ml of TRIS buffer, followed by centrifugation at 1,200 RPM for 5 min. The supernatant was decanted.

After the last washing and centrifugation, 0.4 ml of the developer (60 mg of O-phenylene-diamine (Kodak) and 50 μl of H_2O_2 30% per 200 ml of 0.1 M phosphate buffer) was added to the pellet. After 20 min in the dark the reaction was stopped by adding 0.4 ml of 4 N H_2SO_4 and 2 ml of distilled water to each test tube. A schema of the method is shown in Fig. 1. Absorption was measured by a spectrophotometer at a wavelength of 460 nm and the result was multiplied by 1,000 to avoid decimal numbers. At each assay, 1 test was performed with 0.4 ml TRIS-buffer instead of test-serum to adjust the spectrophotometer to zero (buffer control). The values for the same serum analysed repeatedly in the same assay differed little (< 5%). Also, the differences between the values of any two given sera analysed in consecutive assays showed only slight variation (< 5%). The absolute values for the same sera, however, varied from assay to assay (± 12%). To compensate for these differences, at each assay 1 serum (pooled from 30 healthy mice) was used as reference value (control serum, C). The absorption-values measured for the test-sera (T) were related to the value obtained for the control serum (C) analysed in the same assay by calculating test/control (T/C) ratios.

Statistical analysis. Analyses of covariance were done to test differences in slopes. For analysis of differences in means the Student's t test was used.

Results

Experiment #1. Tumor growth (0.66 +/− 0.33 mm, Fig. 2) was noted 9 days after injecting 5×10^5 MBT cells in the RHL of 33 mice. Tumor size increased until death from tumor cachexia, usually at about 27 days. Serum was sampled until death. Analysis of the sera sampled every 3 to 4 days showed a corresponding increase of the T/C ratio beginning on day 9. The values of the sera from days 0, 3 and 6 were 1.01, 1.03 and 1.03. The 3 results obtained with pooled sera from 9 control mice having received 0.1 ml of PBS in their RHL were 0.95, 1.05 and 0.99 on days 0, 15 and 24, respectively. In mice with growing tumors, the highest value (2.59) was observed at day 24 followed by a decline in the preterminal period. The increase of the T/C ratio in animals with MBT is significantly different when compared to the controls ($p < 0.005$).

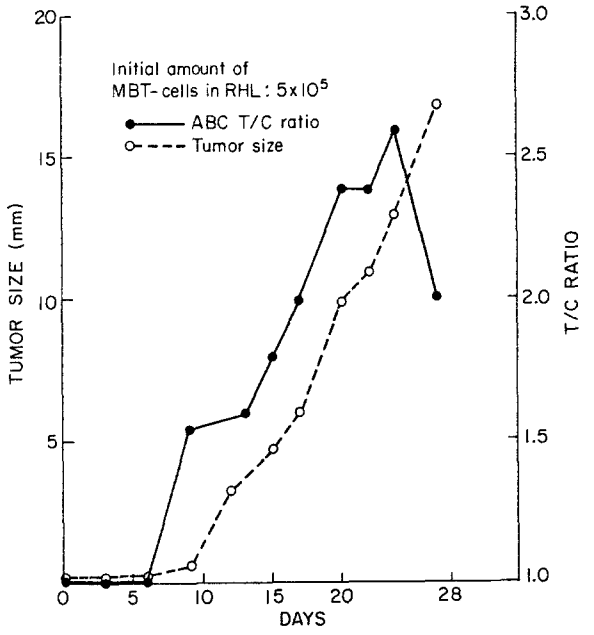


Fig. 2. Experiment #1: Tumor size in mm and T/C ratios measured after injection of 5×10^5 MBT cells on day 0 in RHL. T/C ratio in control animals was between 0.95 and 1.05. The increase of the T/C ratio in animals with MBT is significantly different when compared to control animals ($p < 0.005$)

Experiment #2. To determine the serum levels after removal of the MBT-tumor from the RHL, the legs were amputated under general anesthesia (Nembutal ip) 2 weeks following injection of 5×10^5 MBT cells or 0.1 ml PBS in the RHL (27 mice per group). The results are summarized in Table 1. Whereas the T/C ratios of the 6 pooled sera sampled at different times from the 18 control mice remained between 0.95 and 1.0, tumor-bearing mice had an increased T/C ratio of 1.29 at the time of surgery and 6 days later it was found to be 1.44. Unexpectedly, 13 days after amputation of the RHL, 3 mice showed macroscopic recurrences. They were bled and the value of the pooled sera (1.69) was higher than in 3 other animals sacrificed the same day and not showing macroscopic recurrence (1.38).

Three weeks after amputation, no other animals had visible recurrences. However, 5 weeks after tumor removal, 3 of the remaining 6 mice had macroscopic evidence of recurrences and their T/C ratio (1.8) was again higher than in those with no recurrences (1.24). The differences of serum values observed at day 13 and day 34 between animals with or without recurrences is significant ($p = 0.05$).

Experiment #3.1. To determine whether the rise of the T/C ratio according to time is caused by the increased tumor size or by the duration of exposure to the tumor, 4 groups of mice (total $n = 78$) were simultaneously injected with different amounts of MBT in the RHL. The lowest number of 5×10^3 tumor cells was increased by a factor of 10, resulting in a maximal number of 5×10^6 for the fourth group. Figure 3a shows the different tumor sizes according to the initially injected number of MBT cells. The T/C ratios show the same pattern (Fig. 3b). An elevated serum value is first observed in animals with the highest initial tumor cell number. However, for a given tumor size (e.g., 10 mm), animals with an initially lower cell number showed later comparable T/C ratios. Sera of 9 control animals at the time of PBS injection and 4 and 6 weeks thereafter had a T/C ratio of 1.04, 1.07 and 1.13 respectively.

Experiment #3.2. Two additional groups of mice ($n = 36$) were injected with irradiated cells. In the first group, 5×10^5 cells were injected in the RHL and in the second group 5×10^7 cells was given. After 2 weeks, only mice with the higher initial amount of irradiated MBT cells showed evidence of tumor (1 mm nodules). Later, no macroscopic changes could be observed at the sites of injection. Figure 4 shows that the serum values were always higher in the mice with the initially higher number of injected irradiated cells. This difference is significant ($p < 0.01$). The peak value occurred in both groups two weeks after inoculation. The T/C ratios after one and two weeks were significantly higher ($p < 0.05$) than in any of the 4 previously described groups of mice with growing tumor (experiment #3.1, Fig. 3a/b).

Experiment #4. 72 mice (18 mice per group) were injected at 4 different sites with 5×10^4 MBT cells (Table 2). The value for tumor growth in muscle was taken from experiment #3.1. Unexpectedly, after subcutaneous injection, the

Table 1. Experiment #2. T/C ratios in mice undergoing surgical removal of the tumor 14 days after injection of 5×10^5 MBT in RHL. Controls with 0.1 ml of PBS in RHL. The differences of serum values observed at day 13 and day 34 between mice with or without recurrences is significant ($p = 0.05$)

	At time of tumor inoculation	At time of tumor removal	6 days after tumor removal	13 days after tumor removal	21 days after tumor removal	34 days after tumor removal
	0.99	1.29	1.44			
No macroscopic evidence of recurrence				1.38	1.35	1.24
With macroscopic evidence of recurrence				0.97		1.80
Controls	0.99	0.95	0.98	0.97	0.98	1.0

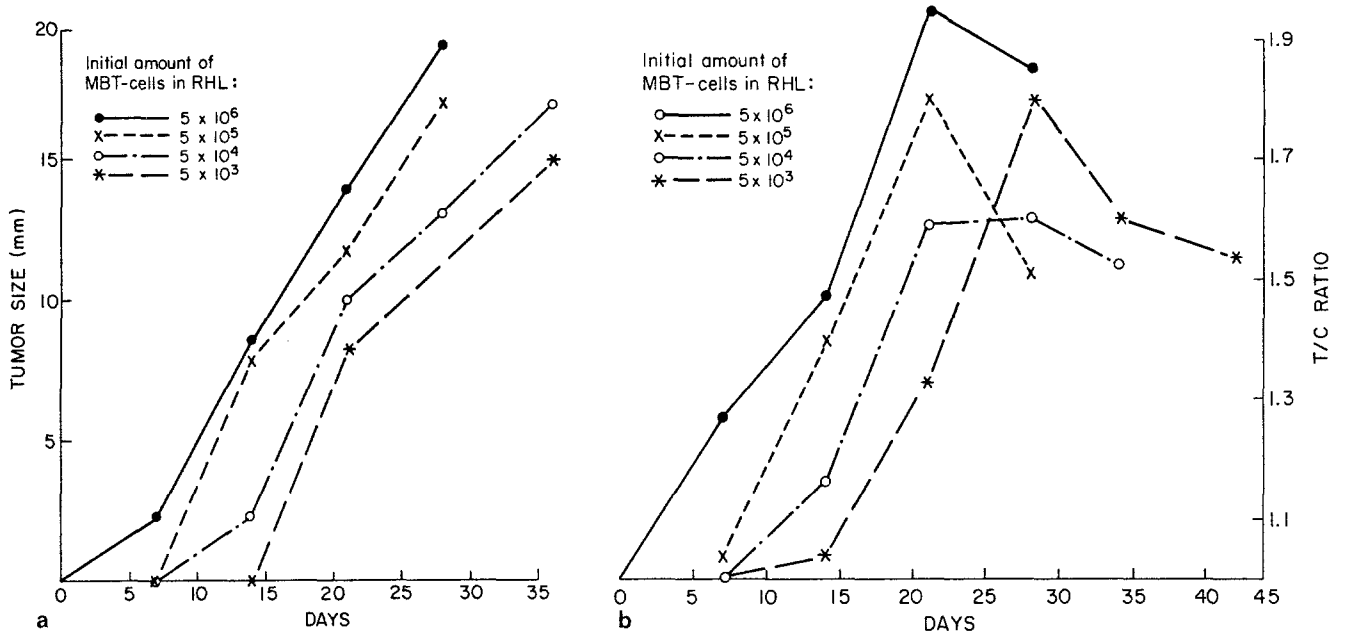
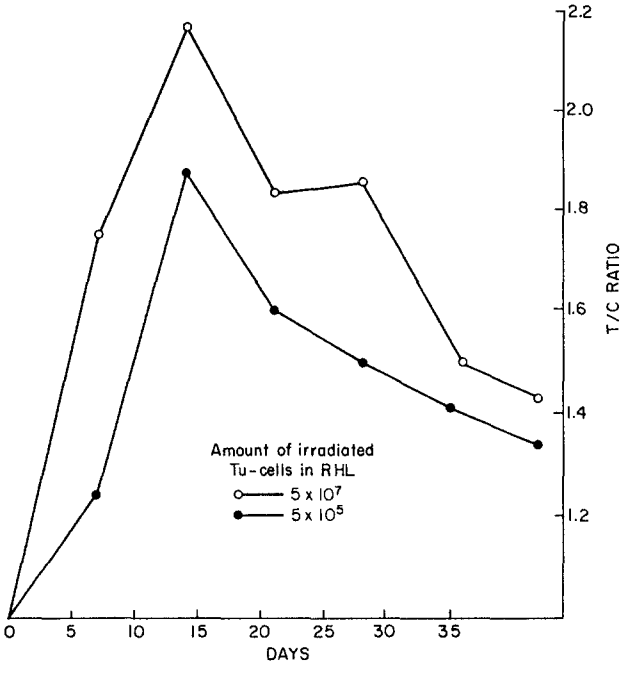


Fig. 3a/b. Experiment # 3.1. Differences of tumor size (a) and T/C ratio (b) according to time and initial amount of injected tumor cells. Comparable tumor sizes show corresponding T/C ratios



tumor grew best in the underlying muscle. After intraperitoneal injection, only in 3/18 mice was a tumor visible in the peritoneal cavity. In the majority, no tumor was found or, after 3 to 4 weeks, tumor grew in the muscle of the abdominal wall at the injection site. There were similar findings after transcutaneous injection of tumor cells into the liver – only 4/16 mice had macroscopic tumor in the liver. Tumor growth in the lung was found in only 1/18 mice, though 7 had tumor in dorsal muscle at the site of the previous transcutaneous injections. The remaining mice had no macroscopically detectable tumor at various times after tumor injection. 12 control mice had PBS injected into the lung. The mean T/C ratios of their serum values were 1.08, 1.05, 1.01 and 1.02 at the beginning of the experiment, 2, 4 and 6 weeks later. Since serum values were in-

◀ Fig. 4. Experiment # 3.2. Injection of irradiated MBT cells in the RHL. The T/C ratio is significantly higher in mice with a higher initial cell number ($p < 0.01$). The values after 1 and 2 weeks are significantly higher ($p < 0.05$) when compared to animals with growing tumors (Fig. 3a/b)

Table 2. Experiment # 4. T/C ratios in mice with MBT tumors of comparable size (5–8 mm) at different sites 2 to 4 weeks after injection. Not all results from pooled sera (see text)

	Localization				
	Subcutaneous	Intraperitoneal	Lung	Liver	Muscle
T/C ratio	1.46	1.42	1.59	1.34	1.3

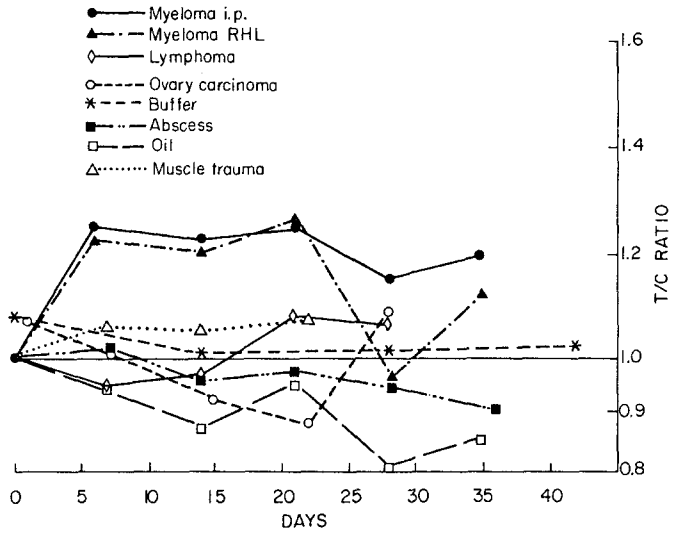


Fig. 5. Experiment # 5. Evaluation of the specificity of the modified ABC method: The T/C ratio is not significantly raised in animals having had repeated mechanical muscle trauma, injection of irritating agents or lymphoma respectively, ovary carcinoma cells when compared to controls ($p < 0.05$). Injection (ip or RHL) of 10^4 myeloma cells deriving originally from Balb C mice into C3H/He mice results in significantly elevated T/C ratios with a maximum of 1.25 ($p < 0.0001$). *Note:* The baseline (T/C ratio 1.0) is higher in comparison to all other figures

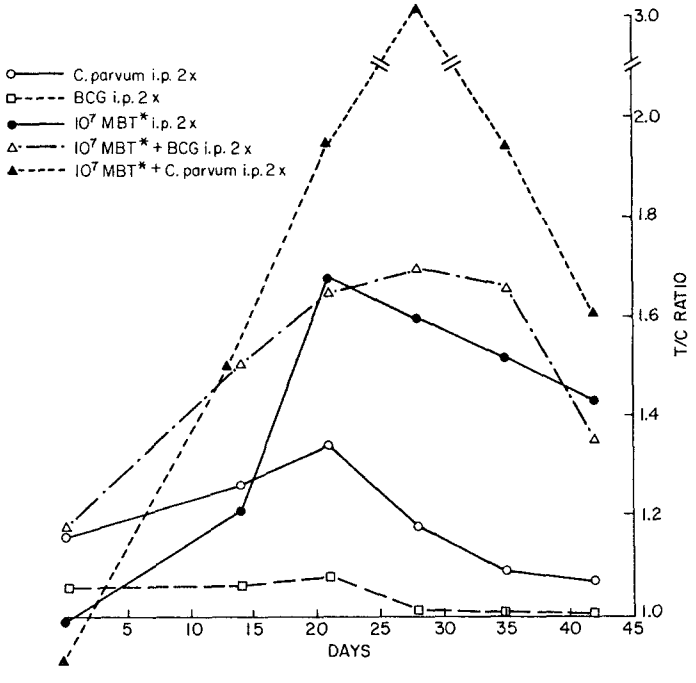


Fig. 6. Experiment # 6.1. Effect of immunomodulators on the T/C ratio. High levels are observed in the 3 groups of mice immunized with irradiated MBT cells. The addition of *C. parvum*, which itself induces a significant rise of the T/C ratio to 1.34 after 3 weeks ($p < 0.0001$), provokes an additional increase of the T/C ratio to a maximum of 3.14. BCG does not influence the values, if given alone or in combination with irradiated MBT cells

cluded only from mice in which macroscopic tumor developed in the organ in which tumor cells were injected, the T/C ratios shown in Table 2 are partially based on sera from 1 or 2 mice only. The values are all significantly elevated ($p < 0.001$).

Experiment # 5. As shown in Fig. 5, injection into the RHL (of 18 mice per group) of mineral oil, mycelium of *Aspergillus fumigatus* or 10^3 cells of a rapidly growing lymphoma did not cause a significant rise of the T/C ratio. Repeated mechanical injuries to the RHL or intraperitoneal growth of ovarian carcinoma caused no increase compared to the values of 9 control mice ($p < 0.05$). A significant rise of the T/C ratios to values around 1.2, however, was found after injecting 5×10^4 myeloma cells (originally derived from Balb C mice) into RHL or intraperitoneally ($p < 0.0001$). Macroscopic tumor growth (1.4 cm, histologically confirmed) in the abdominal wall was observed in 1/36 mice, which was bled separately (T/C ratio 1.13). Nevertheless, the characterisitic of the myeloma slopes (+/– horizontal, Fig. 5) when compared to the slopes observed in animals with growing tumors (Fig. 3b) differs significantly ($p < 0.05$).

Experiment # 6.1. The intraperitoneal injection (repeated after 3 days, 18 mice per group) of immunomodulators alone resulted in an increased T/C ratio when irradiated MBT cells were used. The level was further increased by addition of *C. parvum* (with the highest T/C ratio at 3.14, Fig. 6). *C. parvum* alone also provoked a significant elevation of the T/C ratio (1.34) ($p < 0.001$). The same amount of BCG, however, did not cause an elevation of the T/C ratio nor did it show, unlike *C. parvum*, an additional rise of the T/C ratio when combined with irradiated MBT cells.

Experiment # 6.2. The influence of various methods of immunomodulation on the T/C ratio was measured when viable MBT was present. Animals ($n = 18$ per group) inoculated with 5×10^5 living MBT cells in the RHL were treated (i.p.) with one of the following on days 0 and 3: *C. parvum*, 700 µg; 10^7 irradiated MBT cells; BCG 700 µg. Tumor growth was significantly retarded ($p < 0.003$) by the irradiated cells and by *C. parvum* at 2 and 3 weeks, as compared to tumor growth in untreated animals and those receiving BCG (Fig. 7a). In animals receiving BCG, tumor sizes were comparable to those given no immunomodulator. After 2 and 3 weeks the T/C ratios of the serum values also differed significantly ($p < 0.003$), according to the size of the corresponding tumor (Fig. 7b). The serum values were not substantially altered by the immunomodulators when a growing tumor was present. In the preterminal state (28 days), the T/C ratios were low, as observed in the previous experiments.

In all the experiments, a total of 16 serum samples derived from 48 control mice were analyzed. The mean of these T/C ratios was 1.01 (0.95–1.13). The absolute values for the same control serum C varied from 327 to 441 (mean 374, SD ± 34) in 9 different assays.

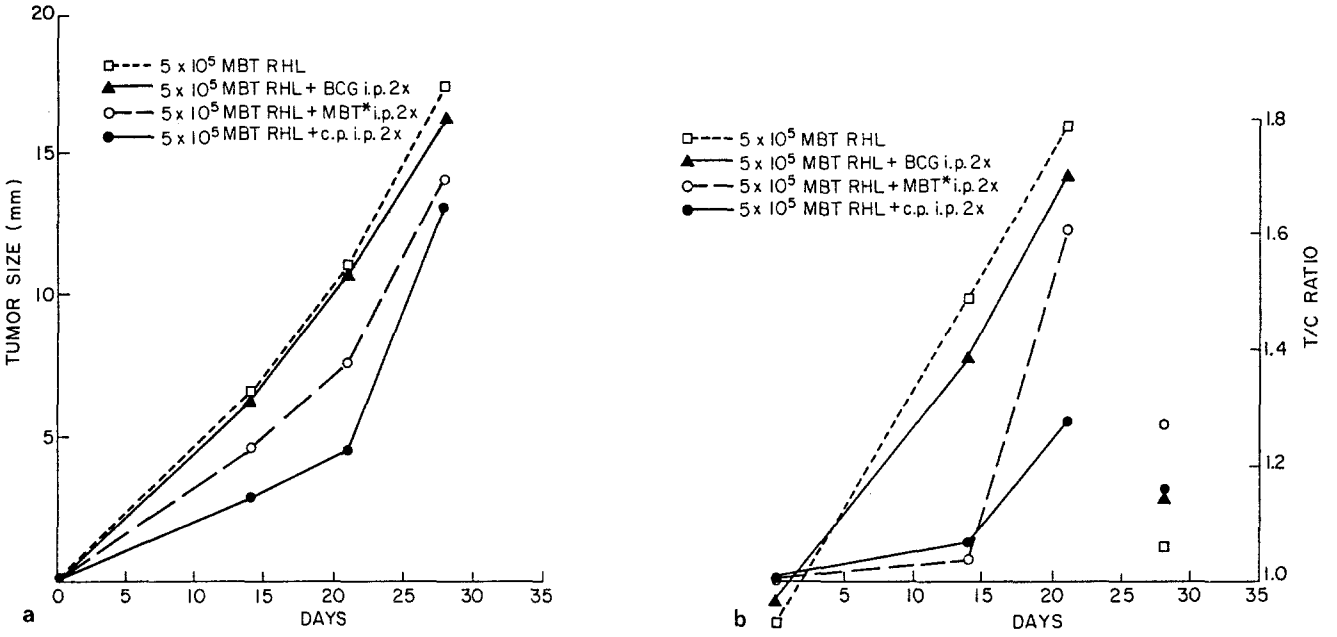


Fig. 7a/b. Experiment # 6.2. Intraperitoneal injection of irradiated MBT cells or *C. parvum* retards significantly ($p < 0.003$) tumor growth when compared to animals with intraperitoneal BCG or 5 x 10^5 MBT in RHL alone (7a). The T/C ratio corresponds to the various tumor sizes, and, in the presence of growing MBT tumor, is not substantially influenced by the immunomodulator itself

Discussion

The method for detecting and quantitating serum factors related to the presence of MBT cells in mice is in principle based on a combination of established immunohistochemical techniques using 2 antibodies, peroxidase and a chromogenic substrate. With the ABC method, an enhanced sensitivity is obtained, quite similar to the peroxidase-antiperoxidase (PAP) technique. However, the affinity of avidin to biotin is stronger than in an antigen-antibody reaction [8]. The use of a water-soluble dye (analogue to the enzyme-linked immunosorbent assay, ELISA) makes an objective measurement of the absorption by a spectrophotometer possible and thus avoids time-consuming microscopic evaluations with the inherent subjective interpretations of the results.

Contrary to most other immunoassays performed on cells derived from tissue culture cell lines, the procedure described herein avoids fixation of the cells in order to reduce iatrogenic changes in the antigenic structure of the target cells. The extent to which this might be important requires further evaluation. Because the cells are unfixed, their number must be relatively high in order to have sufficiently large pellets, allowing the change of reagents and washings without losing the target cells. Preliminary tests have shown that a decrease in target cells, though desirable for technical reasons, would imply further reduction of the amount of test serum by higher dilutions, thus causing an increased possibility of error. It is crucial that the number of target cells is high enough to ensure an excess of antigens. In this regard, the method described differs from other immunohistochemical assays used to detect surface antigens. To quantitate these latter antigens, the quantity and type of first and second antibodies, which must be in excess, are known. If, as in our test, the tumor-associated serum components (first antibody) are to be determined, the number of target cells serving as a homogeneous amount of antigens must be in excess.

The nature of the serum factors found in mice exposed to irradiated or living MBT cells and causing the specific rise in the serum values is not known. Although using an anti-mouse anti-IgG as a second antibody suggests that the tumor-associated immune response detected by this modified ABC method is an immunoglobulin or immunocomplex, additional work is needed to assess its characteristics. By using an anti-IgG, additional nonspecific, non-MBT-associated fractions of the serum would be expected to react with the target cells. This explains the elevated values (approximately 350) observed in sera from healthy control mice, compared to the buffer controls in the assay. Nevertheless, the significant rise of the values observed in sera from mice exposed to MBT cells (living or irradiated) suggests that additional, probably tumor-related reactions, occur with the involvement of tumor-associated antigens, antibodies or antibody complexes. This is supported not only by preliminary findings, showing that double the amount of serum from healthy mice produced a relatively modest rise in values (437, corresponding to a T/C ratio of 1.25), but also by experiment # 5. Repeated muscle necrosis with hematomas and scarring, the presence of an abscess, or granulomatous inflammatory reactions did not produce elevated levels in the test results, nor did the small number of other tumors tested. Only the injection (ip or im) of myeloma cells originally derived from a different strain

which might be expected to induce a host-versus-graft reaction (only 1/36 mice showed a detectable tumor growth), increased the T/C ratio to a maximal value of 1.25. This increase is significant, compared to the other values shown in Fig. 5. However, the slope differs significantly and it is still low compared to values observed in mice with MBT tumors.

Unlike BCG which had no influence on the T/C ratio, in doses and in the strain used in this experiment, the repeated intraperitoneal injection of the biological response modifier *C. parvum* caused a maximal T/C value of 1.34 (Fig. 6). These findings indicate that the test results can be influenced by stimulating the mouse immune system. However, in the presence of a growing tumor, the results have not been substantially falsified by the immunomodulators used in our experiments (Fig. 7b).

The T/C ratios correspond to the tumor size. Absorbance of circulating factors induced by the growing tumor may occur. This is supported by the significantly higher T/C ratio after 1 and 2 weeks when irradiated cells are given (Fig. 4), compared to any other group of mice (from the same subclass) with growing tumors (Fig. 3b).

Further evidence that the elevation of the T/C ratio was caused by a tumor-associated immunoreaction was supported by findings in human tumors when the same method of analysis was used in our laboratory. Sera derived from patients with kidney or bladder cancer were indistinguishable from healthy donors when exposed to other than the corresponding tumor cell line (submitted for publication).

Variations in the absorption values for the same serum sample (control serum, C) among the different test series with an SD +/− 34 and a mean value of 374 (despite calibration of the spectrophotometer according to the concurrent buffer controls) can be caused by various factors: differences in target tumor cells (age, preparation); alterations in the total cell surface caused by clotting of the cells; loss of cells during washing and resuspension and minor differences in the final concentrations of the highly diluted reagents and sera. However, the differences are small enough to allow compensation by calculating the ratios between test serum and standard control serum (T/C ratio). The reliability of the method is also expressed indirectly by the small differences of T/C ratios measured in the 16 different serum samples derived from the various groups of control mice with a mean value of 1.01 (0.95–1.13).

Taking only pooled blood from 3 randomly selected mice for every analysis is an absolute minimum when considering the individual differences in tumor uptake, immune response and general condition of the mice. This may explain the minor differences found between the experiments, or within the different controls (Fig. 5). For example, the tumor size 2 weeks after injection of 5×10^5 MBT in RHL was 2 mm smaller in Fig. 7a compared to Fig. 3a. The higher T/C ratios for a given tumor size shown in Fig. 2, compared to Fig. 3b or 7b (same initial amount of MBT cells) could also be caused by the difference of mouse strain. On the other hand, our results show that the humoral immune response is measurable in various C3H strains. The consistent decline of the T/C ratio in the preterminal phase is explained by a compromised immune system in cachectic, moribund mice.

Injection of MBT cells into various organs caused a rise of the serum values regardless of where the tumor grew. However, since many mice did not show tumor growth at all, or only along the path of injection (suggesting a high affinity for muscle tissue), no definite conclusions concerning the T/C ratio for MBT size according to organ site can be drawn. Moreover, we do not know how the serum levels change after removal of the tumor, since there were many recurrences. Nevertheless, these recurrences were heralded by elevated serum levels, pointing to the potential diagnostic use of the assay.

Although various factors have been found to influence the results of this assay, it is possible to monitor the growth of an MBT by the modified ABC method. Sensitivity, specificity and reliability of the test are high. Based on these findings, it seems justified to develop this method further and assess its use for diagnosis of human cancers.

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