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ORIGINALS

Effect of Intralesional and Systemic BCG-Application or a Combined Cyclophosphamide/BCG Treatment on Experimental Bladder Cancer

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Summary. Infiltrating transitional cell carcinomas of the urinary bladder were induced by ingestion of 0.188% N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) in 145 female Wistar rats. After 8 months of carcinogen exposure, the animals were divided into different treatment groups. They received cyclophosphamide intraperitoneally as a single injection or BCG either once intralesionally or weekly subcutaneously or a combination of cyclophosphamide followed by subcutaneous BCG. The treatment effect was determined by body weight measurements and bladder tumour weight after 12 months. Compared with a control group statistically significant differences of bladder tumour weights were found after treatment with BCG alone or in combination with cyclophosphamide. Intralesional BCG resulted in an insignificant increase of tumour weights.

Key words: Experimental bladder carcinoma, Cyclophosphamide, Tumour weight.

The reliable induction of urinary bladder carcinomas can be achieved in rats by feeding with 0.188% N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) (13, 15, 16, 24, 33, 45, 47, 48). The morphological changes during carcinogenesis as well as the biological behaviour of this tumour have been evaluated recently (1, 26).

Since chemotherapy of FANFT-induced bladder carcinomas with cyclophosphamide was shown to yield encouraging results in mice (14, 41), this substance may be assumed to be effective in our experimental model. To our knowledge, no attempt has been undertaken until now to treat chemically induced bladder tumours with BCG, either systemically or intralesionally. Furthermore, combined treatment with cyclophosphamide and BCG may result in a synergistic therapeutic effect, particularly because in other experimental tumours this type of chemoimmunotherapy has proved to be most effective (2, 12, 30, 34, 35). Our investigations were performed to study the value of various treatment protocols with both cyclophosphamide and BCG alone and in combination.

MATERIAL AND METHODS

145 female Wistar rats (Han/Bö) aged about 35 days, weighing 70-90 g were used. Further details of tumour induction and documentation of cancer development by a transillumination technique were exactly as reported previously (1). Cyclophosphamide was purchased from the Asta Company, Bielefeld (Germany) and BCG from the Research Foundation, Chicago, Illinois (USA). All animals were weighed monthly. At the time of first tumour appearance after 8 months of carcinogen exposure, the rats were divided into the following treatment groups:

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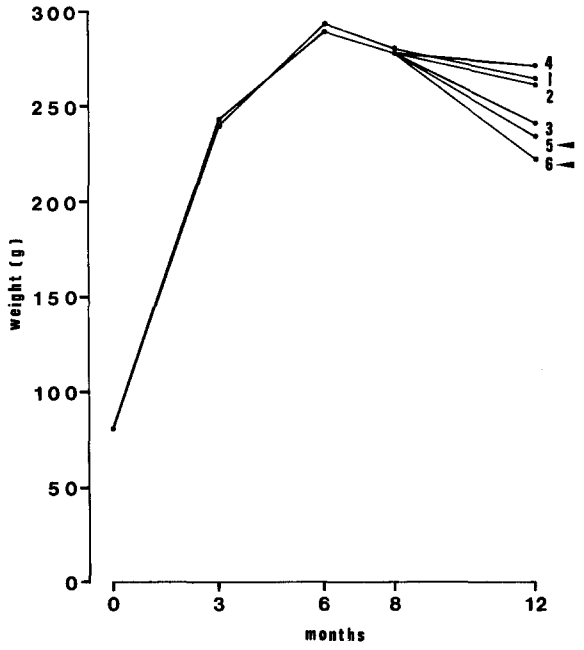

Fig. 1. Body weights of experimental rats during carcinogenesis and after application of different treatment protocols.
1 = group 1 (NaCl-treated control)
2-6 = groups 2-6 (different treatment protocols)
▬ = significant difference to group 1 ($p < 0.01$)

Group 1 (NaCl s.c.): 20 rats received weekly 0.1 ml saline subcutaneously (s.c.) into the inguinal areas alternating between the right and left sides.

Group 2 (Cycl. i.p.): 20 rats received one injection of 100 mg cyclophosphamide per kg body weight intraperitoneally.

Group 3 (BCG i.l.): 20 rats received 0.1 ml of a freshly prepared BCG vaccine (approximately 2 x 10^7 bacilli) through the intact bladder wall into the tumour nodule. This injection was performed in cooperation with the last transillumination procedure.

Group 4 (BCG s.c.): 15 rats received weekly 0.1 ml of the BCG vaccine subcutaneously into the inguinal areas alternating between the right and left sides.

Group 5 (Cycl. i.p. + BCG/I s.c.): 25 rats received one injection of 100 mg cyclophosphamide per kg body weight followed after one week by weekly subcutaneous BCG injections into the inguinal areas alternating between the right and left sides.

Group 6 (Cycl. i.p. + BCG/II s.c.): 25 rats received the same treatment as group 5 except that the weekly BCG injections started 2 weeks after cyclophosphamide application.

Before the end of the experiments, up to 9 animals died in each group. However, the causes of death could not be determined due to the cannibalism of the surviving animals in the same cage. At the termination of the experiments after 12 months, all animals were sacrificed and a complete autopsy of each rat was performed. All tumour containing urinary bladders were extirpated and the weight of each bladder-tumour specimen was determined. In addition, all kidneys as well as those lungs exhibiting gross pathological findings were extirpated. The organs were fixed in 10% buffered formalin, embedded in paraffin and stained with haematoxylin-eosin.

Histopathological staging of the bladder tumours was performed according to the Marshall-Jewett classification and the 3 grades of malignancy were determined by applying the criteria of Mostofi et al. (32) and Koss (27) (for further details see Kiel et al. (26)).

Statistical assessment of the relationship between tumour stage, weight and grade was performed by contingency table analysis. The median values of body weight and bladder tumour weights were compared by the T-test or variance analysis.

RESULTS

Following 8 months of FANFT exposure gross tumours were present in all urinary bladders examined. Histological examination showed infiltrating transitional cell carcinomas with papillary growth.

Figure 1 represents the median body weights of the rats in the control group 1 and the differently treated groups 2-6. For the first 6 months of FANFT exposure the body weights in all experimental groups increased after which time the weights showed a tendency to fall. At

Table 1. Bladder-tumour weights

Animal group	Bladder tumour weights (g)		Significant difference to group 1
	Median	Range	
Group 1 (NaCl s. c.) N = 14	3.120	3.180	-
Group 2 (Cycl. i. p.) N = 14	2.665	4.830	-
Group 3 (BCG i. l.) N = 14	4.590	19.590	-
Group 4 (BCG s. c.) N = 10	1.380	3.060	$P < 0.05$
Group 5 (Cycl. i. p. + BCG/I s. c.) N = 16	0.935	2.790	$P < 0.01$
Group 6 (Cycl. i. p. + BCG/II s. c.) N = 18	0.975	2.310	$P < 0.01$

Table 2. Stage and grade of FANFT-induced urinary bladder tumours

Animal groups	Tumour stage					Significant difference to group 1	Tumour grade			Significant difference to group 1
	I	II	III	IV	V		1	2	3	
Group 1 (NaCl s. c.) N = 14	-	7	4	3	-	-	-	10	4	-
Group 2 (Cycl. i. p.) N = 14	-	9	5	-	-	-	-	11	3	-
Group 3 (BCG i. l.) N = 14	-	6	8	-	-	-	2	7	5	-
Group 4 (BCG s. c.) N = 10	-	7	3	-	-	-	-	8	2	-
Group 5 (Cycl. i. p. + BCG/I s. c.) N = 16	-	13	3	-	-	-	4	9	3	-
Group 6 (Cycl. i. p. + BCG/II s. c.) N = 18	-	12	5	1	-	-	9	7	2	-

the termination of the experiments, the median body weights in the groups 5 and 6 differed significantly ($P < 0.01$) from those of the NaCl-s. c. treated control group 1.

Evaluation of the bladder tumour weights revealed statistically significant lower values in treatment groups 4, 5, and 6 (Table 1). On the contrary, intralesional BCG application results in a statistically insignificant increase of bladder tumour weights. No effect was noticed after injection with 100 mg cyclophosphamide alone.

Each bladder tumour specimen was histologically differentiated according to tumour stage and grade. The results of this assessment for the groups 1-6 are presented in Table 2. There was no statistical difference of tumour stage and grade between control and experimental animals.

Morphological alterations of the renal pelvis

Table 3. Histological findings in the kidneys of the experimental animals after application of different treatment protocols at termination of the experiments

Animal groups	Histological findings in the kidney			
	Normal	Dysplasia	Transitional cell carcinoma	hydro-nephrosis
Group 1 (NaCl s. c.) N = 14	18	9	1	2
Group 2 (Cycl. i. p.) N = 14	16	4	2	8
Group 3 (BCG i. l.) N = 14	20	2	2	4
Group 4 (BCG s. c.) N = 10	12	3	1	6
Group 5 (Cycl. i. p. + BCG/I s. c.) N = 16	20	10	-	2
Group 6 (Cycl. i. p. + BCG/II s. c.) N = 18	24	8	4	2

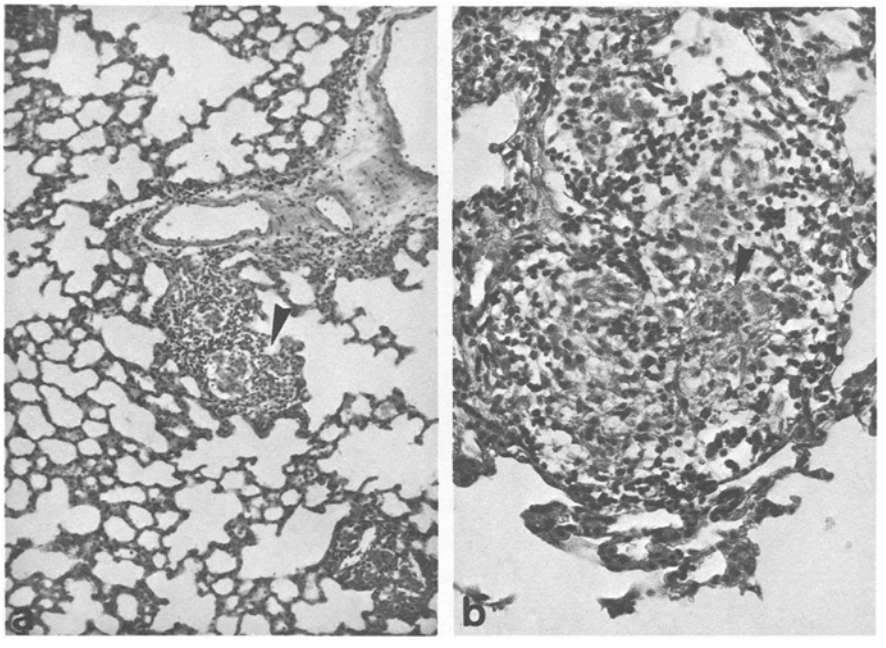

Fig. 2 a and b. Histological changes of the lungs in several BCG treated animals. a) Granulomas predominantly arranged around vessels (➡) x 90. b) Higher magnification with epithelioid cell granuloma - containing a multinucleated giant cell (➡) x 250

urothelium due to FANFT ingestion ranged from normal findings to dysplasia and infiltrating transitional cell carcinomas. In addition, various degrees of hydronephrosis and pyelonephritis were present. The histological findings in the kidneys of the groups 1-6 are summarised in Table 3. No correlation exists between the different treatment protocols and the development of dysplasia and transitional cell carcinoma in the renal pelvis. In addition, the incidence of hydronephrosis did not correlate with any parameter of the bladder tumours, such as weight, stage and grade (compare Table 3 with Table 2).

All saline injections were tolerated very well

Table 4. Histological findings in the lungs

Animal groups	Histological findings in the lungs
Group 1 (NaCl s. c.) N = 14	-
Group 2 (Cycl. i. p.) N = 14	-
Group 3 (BCG i. l.) N = 14	epithelioid cell granulomas N = 4
Group 4 (BCG s. c.) N = 10	epithelioid cell granulomas N = 2
Group 5 (Cycl. i. p. + BCG/I s. c.) N = 16	epithelioid cell granulomas N = 1
Group 6 (Cycl. i. p. + BCG/II s. c.) N = 18	epithelioid cell granulomas N = 2

as we did not observe any relevant side effects after cyclophosphamide treatment. Intralesional BCG application did not interfere with the normal process of wound healing. In the treatment groups 4-6 with weekly BCG injections, local inflammatory indurations, and in a few cases abscesses developed in the inguinal fold with a purulent discharge. In a few animals of the BCG treated groups 3-6, autopsy revealed multiple white nodules of about 1-2 mm diameter in both lungs. Histological examination of these lesions showed there to be epithelioid cell granulomas (Fig. 2). The frequency of these findings in the different BCG treated groups is shown in Table 4.

DISCUSSION

Our experimental data confirm and extend earlier results of a 100% induction of transitional cell carcinomas of the urinary bladder in female Wistar rats, after ingestion of 0.188% FANFT (1, 26). Other authors have reported similar findings using Sprague-Dawly (15, 16) and Fischer rats (13, 45, 48).

Prolonged BCG treatment in the groups 4-6 results in a statistically significant reduction of bladder tumour weights. On the contrary, intralesional BCG-application caused an insignificant increase of tumour weight, which may be considered as tumour enhancement. Cyclophosphamide did not influence neoplastic growth, however in combination with subsequent BCG it proved to be quite effective (Table 1).

The therapeutic efficacy of mycobacteria, especially BCG, has been shown for several experimental tumour models (8, 9, 11, 18, 22, 23, 42, 44, 46, 50). The exact mechanism of the anti-tumour action of BCG has not yet been elucidated. Nevertheless, non-specific stimulation of the immune system has widely been accepted as one principal mode of action (6, 7, 19, 21, 31). BCG is most active provided direct contact with cancer cells is possible (4, 6, 8, 23, 50), although regression of distant metastases has also been observed (22, 40). Immunocompetence of the host is a prerequisite for action of BCG (51).

The problem still exists as to which factor determines the immunotherapeutic action of a given BCG-preparation. The number of viable units, virulence of the strain, replicating capacity of the viable units, and physico-chemical characteristics of the bacterium (21, 28, 37, 38) have been considered. The observation of Khalil and coworkers (25) is of special interest because they noticed that the immunostimulating effect of BCG corresponds with the presence of septicaemia. In most assays, fresh vaccine proved to be most effective (21).

There was also a correlation between the loss of activity and the storage temperatures (17). The optimal dosage of BCG for immunotherapy appears to be between 10^7 and 10^8 bacilli/injection. Lower or higher concentrations resulted in a reduction or even loss of therapeutic activity (21, 22, 51).

Whether, in our experimental system, intralesional BCG injection had caused the phenomenon of tumour enhancement, cannot be ascertained. The possibility of such an enhancing effect of BCG, however, has been demonstrated (4, 5, 29, 42, 49).

With reference to the literature, in other experimental tumour models combined cyclophos-

phamide/BCG treatment showed a synergistic effect and proved to be superior to either treatment alone (2, 12, 30, 34, 35). Our results demonstrate that FANFT-induced transitional cell carcinomas of the urinary bladder can also be effectively treated by this protocol. The rationale for this effect probably lies in a relative selective inhibition of the B-lymphocyte dependent humoral antibody response by cyclophosphamide (10, 20, 36, 43). Thus, the formation of antigen-antibody complexes may be prevented or diminished. These complexes, in turn, are known to exert an enhancing effect on the tumour growth due to specific blockade of tumour surface antigens (3, 39). Most authors emphasize that chemo-immunotherapy is most effective if a defined time gap between each treatment is observed (9, 12, 34). On the contrary, our experimental results display that a time interval of either one or two weeks does not significantly influence the treatment results.

Apart from its action in combination with BCG, cyclophosphamide alone may exhibit a significant chemotherapeutic effect on FANFT-induced bladder tumours after a prolonged period (14, 41). Our experimental data show, however, that a single dose of cyclophosphamide does not significantly reduce the total tumour mass.

At the termination of the experiments after 12 months, no treatment modality had resulted in a statistically significant effect on either tumour stage or grade. Despite of the reduction of tumour weight in the groups 4-6, the depth of tumour infiltration had not been altered either by cyclophosphamide or BCG.

The occurrence of dysplasia and infiltrating transitional cell carcinoma of the renal pelvis after FANFT ingestion has also been reported in the literature (15, 16).

The side effects of the different treatments reflect a most interesting problem. Except for the deaths of several animals, the loss of weight in all groups may be attributed to tumour growth as well as therapy effect. Most conspicuous is the marked weight loss in the groups 5 and 6 with the most effective tumour reduction by cyclophosphamide and BCG (compare Fig. 1 with Table 1). In addition the presence of epithelioid cell granulomas in several lungs of BCG treated animals represent another side effect of BCG treatment.

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