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A Follow-Up Study of Urinary Bladder Patients Tested for Tumour-Related Lymphocyte-Mediated Cytotoxicity

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Summary. *A total of 143 patients with transitional cell carcinoma of the urinary bladder were tested for lymphocyte-mediated cytotoxicity against the bladder carcinoma cell line T24. Some of the patients were also tested against MANO (another cell line of transitional cell bladder carcinoma origin), HCV29 (from bladder epithelium, probably transformed in vitro) and/or HT29 (from a colon adenocarcinoma). The patients were divided into a high- or a low-responder group for each cell line. The patients were followed up and the correlation between a high response in the cytotoxicity tests and survival was evaluated using an adaptation of the Mantel-Haenszel statistics. No significant correlation could be demonstrated.*

Introduction

A disease-related cytotoxicity of lymphocytes from patients with transitional-cell carcinoma of the urinary bladder has been demonstrated in several studies [1, 2, 3, 13, 15]. However, since lymphocytes from both patients and healthy controls frequently lyse various tumour cells without any obvious relationship to disease, a considerable number of patients may be needed to recognize a statistically significant disease-related cytotoxicity in addition to the natural cytotoxicity. In some studies, for example in that of Catalona et al., no bladder tumour-related cytotoxicity has been found [5].

It is important to see if the disease-related or natural cytotoxicity has a prognostic value. Even if this is the case, nothing can be assumed a priori about the effect of such an influence on survival. The most appealing explanation is that the tumour-related cytotoxicity has a beneficial effect on the course of the disease, and thus acts as some sort of partial immunity. On the other hand, the cytotoxicity may have the opposite effect and the cytotoxic reactions could mediate deleterious rather than beneficial auto-immune cell damage. The cytotoxicity may also be disease-related in the sense that it is related to the activity of the disease. Furthermore, the fact that cytotoxicity has different kinds of influence does not mean that the one kind excludes the others in the population of patients.

Essentially, in the present study, the patients investigated for cytotoxicity in the study of Troye et al. [13] were followed up. In contrast to a recently published study by Vilien et al. [16] the present study revealed no correlation between cytotoxicity and patient survival.

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Materials and Methods

Patients. A total of 143 TCC patients with urinary bladder cancer treated by irradiation and surgery at Radiumhemmet and the Department of Urology, Karolinska Hospital, Stockholm, Sweden, were tested for lymphocyte-mediated lysis against T24 cells between December 1974 and October 1977. Their tumours had been classified according to UICC [14] and graded according to the World Health Organization (WHO) grades 1–3. Twenty patients were excluded from the study as the cytotoxic test was regarded as a failure according to the criterion of high background lysis (see below).

Only one of the patients tested had a grade 1 tumour, and she was excluded since progressive disease is seldom seen in grade 1 patients [6]. Two patients aged 84 years at testing had evidently died due to old age and were also excluded. Otherwise, no attempts were made to discriminate between deaths caused by the cancer and deaths due to unrelated causes.

Assay of Lymphocyte Mediated Lysis. Preparation of lymphocytes, target cells used and performance of cytotoxic assay were the same as described by Troye et al. [13]. Briefly, highly purified blood lymphocytes were used in a ^{51}Cr-release assay with the bladder carcinoma cell line T24 [4] as a target. Most patients were tested simultaneously against one or more of a number of cell lines. These included one further bladder carcinoma cell line, MANO, (established by Troye 1974) and HCV29 from a supposedly normal specimen of bladder epithelium, but the latter cell line had also transformed into tissue culture and showed a growth pattern similar to that of malignant cells. Most patients were also tested against HT29, a CEA-producing cell line from a colon adenocarcinoma. The percentage of isotope released into the medium was used as a measure of cytotoxicity, corrected by subtracting the percentage of isotope released in lymphocyte-free controls with only target cells. Spontaneous lysis greater than 25% was considered to be a technical failure and such data were disregarded.

Statistical Analysis. On the basis of results from the cytotoxic assay, patients were divided into a high- and a low-responding group of equal size. The high-responder group was arbitrarily chosen to be the exposed group. The data were investigated for conceivably confounding factors that might suggest that stratification should be carried out according to age and tumor stage. Point estimates of relative risks were calculated by an adaptation of the Mantel-Haenszel statistics [8] for follow-up

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data [7, 10]; 95% confidence intervals were calculated as suggested by Miettinen [9], and checked by the method of Nurminen [11], which gave very similar results.

Results

Numerous factors may influence both the cytotoxic capacity of the patients and their survival, and thus act as confounders. In this study, data were available for the following conceivable confounders: age, sex, treatment at the beginning of the study, stage of tumour and grade of tumour. As expected, age, tumour stage and tumour grade were correlated with survival, while no such correlations were found for sex or treatment at the beginning of the study (Table 1). It should be pointed out, however, that the notation "not irradiated" and "irradiated" refers to the day on which the cytotoxicity test was performed, and that most patients in the "not irradiated" group started treatment shortly afterwards. The influence of the factors in Table 1 was less pronounced for the ratio of high to low responders against T24. The most impressive cross-ratio was obtained for tumour stage and this factor was then used for stratification into two groups of equal size. Although the age of the patients gave rise to a cross-ratio that deviated less from unity, we considered it reasonable to use age as a basis for stratification, too.

The patients were allocated to a high-responder group against T24 if their cytotoxicity was greater than the median (9.0% corrected cytotoxicity), otherwise they were allocated to the low-responder group. The resulting figures are presented in Table 2. The relative risk varied from stratum to stratum. The overall estimation of relative risk was 1.4 (95% confidence interval 0.9, 2.3). The elevation in the relative risk came completely from the strata of patients of advanced aged, where a high proportion of the deaths probably occurred due to causes other than bladder carcinoma. For this reason we attribute the deviation in relative risk from the null hypothesis to chance.

Lymphocyte cytotoxicity was also tested against other cell lines to an extent limited by the lymphocyte yield at preparation and sometimes by technical failures, as already

Table 1. Influence of various factors on the ratio between high- and low-responders against the bladder carcinoma cell line T24 and on mortality

Factor	No. of patients	Cytotoxicity against T24		Mortality	
		Ratio: No. of high-/no. of low-responders	Crossratio	Incidence	Relative risk (95% confidence limits)
Age					
Born in 1907 or before	(60)	0.81	0.71	0.29	2.0 (1.3, 3.0)
Born in 1908 or later	(60)	1.13		0.14	
Sex					
Female	(25)	0.92		0.17	
Male	(95)	1.02	0.94	0.21	1.2 (0.7, 2.2)
Treatment at start					
Not irradiated	(72)	1.25		0.19	
Irradiated	(48)	0.71	1.75	0.22	0.9 (0.6, 1.4)
Tumour stage					
T3 + T4	(52)	0.63		0.30	
T1 + T2	(68)	1.34	0.46	0.15	2.0 (1.3, 3.0)
Tumour grade					
G3 + G4	(97)	0.90		0.25	
G2	(23)	1.30	0.69	0.08	3.2 (1.6, 6.4)

Table 2. The relationship between high or low cytotoxicity against the bladder carcinoma cell line T24 and mortality for different strata

		High cytotoxicity		Low cytotoxicity		Relative risk (95% confidence limits)
		Total observation time[a]	No. of deaths	Total observation time[a]	No. of deaths	
Patients born in 1907 or before	Stage T1 and T2	33.7	14	50.9	8	2.7 (1.2, 2.8)
	Stage T3 and T4	22.9	9	49.2	14	1.4 (0.6, 3.2)
Patients born in 1908 or later	Stage T1 and T2	102.8	9	47.7	5	0.8 (0.3, 2.5)
	Stage T3 and T4	25.3	7	41.7	11	1.1 (0.4, 2.7)
Overall						1.4 (0.9, 2.3)

[a] Given as person years per group

Table 3. Relative risk of bladder tumour patients tested for cytotoxicity against MANO, HCV29, and/or HT29 cells. The same stratification was used as in Table 2. Patients with a corrected cytotoxicity above the median were classified as high-responders for the respective cell line

Cell line	No. of patients tested	Median of corrected cytotoxicity	Relative risk (95% confidence limits)
MANO	46	10	0.9 (0.4, 1.9)
HCV29	89	7	1.0 (0.6, 1.7)
HT29	87	3	1.1 (0.6, 1.9)

Table 4. Relative risk of bladder tumour patients. Patients were allocated to a high- or low-responder group by subtracting cytotoxicity against a control cell line (HCV29 or HT29) from cytotoxicity against T24. The same mode of stratification was used as in Table 2.

Subtraction of cytotoxicity	Median of subtracted cytotoxicity	Relative risk (95% confidence limits)
T24 – HCV29	0	0.9 (0.5, 1.5)
T24 – HT29	6	1.5 (0.9, 2.3)

defined. Forty-six of the patients were tested against the bladder carcinoma cell line MANO; the median of the corrected cytotoxicity was 10%. The patients were divided into a high-responder and a low-responder group of equal size as for T24 and the same mode of stratification was used. The relative risk was estimated accordingly to be 0.9 with a 95% confidence interval of 0.4–1.9 (Table 3). Similarly, the patients tested for HCV29 and HT29 were classified as high- or low-responder for the respective cell lines, and the risk ratio was calculated using the same stratification as for the bladder carcinoma cell lines. As seen in Table 3, no correlation was evident between survival and cytotoxic capacity.

It could not be excluded that although high cytotoxicity against T24 had no prognostic value in itself, it could have if the cytotoxicity value was corrected by subtracting the values for a control cell line. The results of this procedure did not reveal any significant improvement in the relative risk (Table 4). As in the case of cytotoxicity against T24, the elevated relative risk for patients allocated to a group with the aid of the subtraction T24 – HT29 came mainly from the strata of elderly patients.

Discussion

Several factors may influence both cytotoxicity and the survival of patients. The cytotoxicity observed against T24 varied only slightly due to age, sex, treatment at the beginning of the study and tumour grade; however, the tumour stage had a more pronounced effect on cytotoxicity. We believe that age in addition to tumour stage influenced cytotoxicity to some extent; therefore, age was also used as a criterion for stratification.

Tumour grade most markedly influenced patient survival with a relative risk of 3.2 (1.6, 6.4). However, this factor was not chosen among the criteria for stratification, since the majority of the patients were of grade 3 at the start of the study, and tumour grade only had a modest influence on cytotoxicity. Instead, tumour stage was found to be more suitable and was selected as the second criterion for stratification. Tumour stage was used to divide the patients into two groups of about the same size (68 patients of stages 1 or 2; 52 patients of stages 3 or 4). There was also a positive correlation between the patients' grade and stage, most grade 2 patients being of stage 1 or 2. At first, treatment at the beginning of the study was also considered, but in life tables constructed after stratification for age and tumour stage, treatment at the beginning could not be seen to have any effect.

The classification of cytotoxic capacity into groups of high-responding or low-responding patients is a source of considerable error in a study of this nature. Firstly, the cytotoxic effects are small and the relative error is substantial. Secondly, we had no explicitly defined point on the scale from low to high cytotoxicity that could be used as a limit. Instead, the median cytotoxicity was used to divide the patients into two groups of equal size.

No effort was made to exclude deaths occurring for reasons not directly connected with bladder carcinoma. The registry data available on the causes of death, based upon death certificates, are low in quality. A retrospective review of the case records would be more reliable, but an element of subjectivity would then be introduced. Therefore, all deaths were included in this study.

Data on cytotoxicity for most of the patients have been reported previously [13]. It was found that the bladder cell lines T24 and MANO were lysed on average to a higher extent by lymphocytes from bladder cancer patients than by lymphocytes from healthy or clinical controls. In contrast, the control cell lines HCV29 and HT29 were lysed to about the same extent, irrespective of whether the lymphocytes came from bladder patients or from any control group. However, T24 was the only cell line used consistently for every patient, so the data were most comprehensive for this cell line. For this reason, we considered the data for T24 to be most favourable for the study of a relationship between cytotoxicity and survival. Our follow-up study resulted in a relative risk of 1.4 (95% confidence limits 0.9, 2.9) with the high-responders chosen arbitrarily as the exposed group. This result was interpreted to be negative, since the overall relative risk was derived from the strata of elderly patients, where deaths due to causes other than bladder cancer are frequent.

Although the cytotoxicity data were more sparse for the other cell lines, MANO, HCV29 and HT29 were also used to assess the correlation between cytotoxicity and survival, but produced consistently negative results (Table 3).

Vilien et al. [15] have found evidence indicating that better disease-related cytotoxicity is obtained if the cytotoxicity against the bladder tumour cell line (Hu 456) is corrected by subtracting cytotoxicity against a cell line from normal urothelium (Hu 609) or cytotoxicity against a cell line from an unrelated tumour (SAOS 2 from an osteosarcoma). They named these types of corrected values "tumour-specific" and "tumour-type-specific" cytotoxicity, respectively. In a follow-up study they found a positive correlation between both tumour-specific and tumour-type-specific cytotoxicity on the one hand and patient survival on the other [16]. When same approach was applied to our target cells, such a positive correlation between corrected cytotoxicity and patient survival could not be confirmed. It should be noted, however, that the two studies differ in several technical aspects, for example target cell lines and methods for cytotoxic assay.

Another issue is the power of the study, i.e., the chance of detecting a possible relationship between some cytotoxicity

index and patient survival, if indeed it exists. Such a relationship can only be estimated in a heuristic way. We believe that in our study a considerable influence of disease-related cytotoxicity on survival should have been detected, since the influence of age, tumour stage and tumour grade could be demonstrated. However, the situation is less favourable in the case of disease-related cytotoxicity, since the risk of misclassifying patients is much larger in this respect. Another complication is that there were few deaths among the younger patients (born in 1908 or later) and that a substantial number of the deaths among the old patients probably occurred due to causes other than bladder cancer. For this reason, our study does not rule out a modest correlation between cytotoxic capability and survival.

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