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# Cellular Immune Function in Patients With Cancer of the Upper Part of the Gastrointestinal Tract

By

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Lymphocytes of 30 patients were examined for cellular immune function one day prior to operation. In 11 patients the tests were repeated six weeks after surgery. In comparison with 26 healthy blood donors the E rosette values were strongly reduced in the patient group. The zymosan-complement rosette counts were significantly lower if percentages were compared, but not significant when they were expressed in absolute numbers. Lymphocyte transformation tests revealed significant differences between the patients and control cases with all three mitogens applied (PHA, ConA, PWM). In the 11 patients with the tests repeated after operation, the effect of autologous plasma on the cultures was also studied. The main tendency was inhibition but in some cases the patients' plasma enhanced the reaction. Killer cell activity by ADCC was significantly lower in patients' than in the healthy donors' lymphocytes in different target-effector cell ratios.

Based on recent knowledge about immune function, numerous studies have attempted to assess the immune capacity of cancer patients. As expected, first it was found to be impaired in most cases [11, 16, 19, 20]. Subsequent studies revealed that the result depends not only on the technique applied but also on the type and stage of the malignant disease [43].

Since the alterations connected with malignancy essentially affect the cellular immune function [9, 13], most investigations were focussed on this aspect of immunological phenomena, either to evaluate the current status or to predict the later course of events.

Cellular immune function may be impaired by a decrease in number or disturbed function of immunocompetent cells, a genetic defect *ab ovo* or, in most cases, in consequence of the malignancy, since the means of defence are gradually exhausted with progression of the disease [24]. The situation is made worse and worse by the presence of blocking serum factors [14, 18] capable to inactivate the functioning cells [17].

Early and radical surgical intervention represents the adequate solution for cancer patients. Incomplete removal of the malignancy creates a different situation for the host defence mechanisms. The aim of the present was a multiple parameter testing of the cellular immune function of patients with cancer of the oesphagus, cardia or the stomach. In a small group of patients follow-up studies were also carried out.

#### **Patients and Methods**

Lymphocytes of patients with malignant tumour in the oesophagus, cardia or the stomach were examined for cellular immune function one day prior to operation. As the combination of applied techniques was not always the same, results are given for each technique separately. In a small group of patients the tests were repeated six weeks after the operation. The most important details of the methods are given as follows.

#### Mononuclear cell suspensions

Fresh human peripheral blood lymphocytes were prepared from heparinized venous blood by separation on Ficoll-Uromiro gradient according to BØYUM [5]. The harvested mononuclear cells were washed twice in medium 199 and the cell count was adjusted to  $10^7$  cells/ml.

#### E-rosette (ER) formation with sheep red blood cells (SRBC)

SBRC and lymphocytes were mixed in a ratio of 40:1 in Parker 199 medium supplemented with 8-9% absorbed fetal calf serum (FCS). The suspension was incubated at  $37^{\circ}$ C for 15 min, centrifuged at 50 g for 15 min and incubated at  $4^{\circ}$ C for 18 h. The pellet was resuspended by gentle shaking and the percentage of rosette-forming cells (RFC) determined. A total of 200 lymphocytes were counted and all lymphocytes binding more than three SRBC were taken as E-RFCs [2].

#### Determination of active rosette-forming cells (A-RFC)

The test was performed similarly as E-RFC, but after centrifuging at 50 g for 5 min, counting was done immediately.

#### $Zymosan-C_3$ rosette formation (ZC-RFC)

This is a rosette indicator system for detecting B lymphocytes by using zymosan particles coated with complement (ZC) [26]. For rosette formation, 100  $\mu$ l of lymphocytes (10<sup>6</sup> cells/ml) were mixed with an equal volume of ZC

suspension (10<sup>8</sup> particles/ml). For a control, zymosan particles without complement were used. Rosettes were defined as cells possessing three or more adhering ZC particles.

## Lymphocyte transformation test with cryopreserved lymphocytes

## 1. Freezing and thawing procedure

 $10^{7}$  lymphocytes were suspended in 1 ml medium RPMI containing 20% pooled human AB serum. All subsequent steps of the procedure were done at thawing ice temperature. The cell suspensions were diluted with an equal volume of medium RPMI containg 20% dimethyl sulfoxide (DMSO). The lymphocyte-DMSO suspension was transferred in aliquots of 1 ml to polyethylene vials. The vials were transferred to the freezer and slowly frozen until -90 °C. The vials were stored in liquid nitrogen until used.

Cells were thewed rapidly by immersing the vials in a 40 °C water bath upon their removal from the linquid nitrogen. The vials were removed from the were completely thawed. The cell suspension (1 ml) was quickly transferred to 2 ml of medium RPMI containing 10% pooled AB serum for diluting the DMSO. The tubes were then spun at 200 g for 10 min. The supernatant was decanted and the cells washed twice more. The cells were then suspended in medium RPMI containing 10% pooled human AB serum and the viability was determined using trypan blue dye exclusion. The viable cell number was adjusted to  $1 \times 10^6$  cells/ml.

## 2. Lymphocyte transformation test (BAIN (3))

Mitogen-stimulated lymphocyte transformation was prepared in microtiter plates (Greiner) using  $2 \times 10^5$  cells in each culture. For measuring the mitotic capacity of the lymphocytes, we used phytohaemagglutinin (PHA), Concanavalin a (Con A) and pokeweed nitogen (PWM), respectively. PHA was used in three concentrations: 5  $\mu$ l, 10  $\mu$ l, and 50  $\mu$ l from the 1 : 10 diluted stock solution. All tests were performed in triplicates.

Culture were labelled with 1 uCi of tritiated thymidine on day 3 for 5 hr and harvested for scintillation spectrometry. Results were expressed as  $dpm/10^6$  cells.

concentrations of 5  $\mu$ l, 10  $\mu$ l and 50  $\mu$ l, respectively, from the 1 : 10 diluted stock solution, ConA: 5  $\gamma$ /ml and 2.5  $\gamma$ /ml, PWM: 5  $\mu$ l from the 1 : 2 diluted stock solution. All tests were performed in triplicate.

Cultures were labelled with 1  $\mu$ Ci of tritiated thymidine on day 3 for 5 h and harvested for scintillation spectrometry. Results were expressed as dpm/10<sup>6</sup> cells.

## Antibody-dependent cellular cytotoxicity (ADCC)

1. Target cells. Fresh human Rh positive erythrocytes were washed twice and  $3 \times 10^8$  cells were labelled with 100  $\mu$ Ci Na<sub>2</sub> <sup>51</sup>CrO<sub>4</sub> and 100  $\mu$ l anti-D serum in 200  $\mu$ l Na-citrate. After 1 h incubation at 37°C the cells were washed twice and given to 1 ml medium 199 supplemented with 10% pooled AB serum.

2. Effector lymphocytes. Heparinized whole human blood was mixed with 1 g carbamide iron powder and incubated at 37°C for 30 min (removal of phagocytes, monocytes or neutrophils). The lymphocytes were isolated in Ficoll-Uromiro gradient as described previously. The lymphoid cell suspensions contained less than 1% granulocytes and 4% monocytes.

3. Cytotoxic assay. Triplicate cultures were set up containing different target/effector ratio, in a total volume of 200  $\mu$ l culture medium 199 supplemented with 10% FCS. Usually  $2.5-5.0 \times 10^4$  target cells per well were used. The number of effector cells was varied downwards from  $1-5 \times 10^6/\text{ml lympho-cytes}$  (See Fig. 3). 100  $\mu$ l aliquots of the supernatants were then removed and counted in a gamma counter. Maximum isotope release was determined by incubating the appropriate number of  $5^2$ Cr labelled erythrocytes in distilled-water instead of culture medium. Spontaneous chromium release was determined by incubation of target cells without effector cells. The percentage of  $5^1$ Cr release was calculated and the results were expressed as the cytotoxic index (CI):

 $CI = 100x \frac{{}^{51}Cr \text{ release by lymphoid cells } - \text{ spontaneous release}}{\text{maximum } {}^{51}Cr \text{ release } - \text{ spontaneous release}}$ 

## Results

#### Rosette techniques

E-rosette determination of T lymphocytes (ER) and zymosan-complement rosette (ZCR) counting for estimation of B lymphocytes of 22 patients are shown in Table I, in percentages and in absolute cell counts. In comparison with the values for healthy blood donor lymphocytes, the number of ARFCs was significantly less in the patient group. With ZC rosetting there was a significant difference only between the results expressed in percentage. The ratio of lymphocytes taking part in immediate(active) rosette-formation was the same in the patients and the controls.

# Lymphocyte transformation tests

In 30 patient <sup>3</sup>H-thymidine uptake of lymphocytes stimulated by PHA, ConA and PWM is shown in Fig 1. All transformation values represent the maximum reponse of parallel cultures with three different mitogen concentra-

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#### TABLE I

Rosette test formation with lymphocytes of healthy blood donors and tumour bearing patients

	Number of absolute lymphocytes	Number of E-rosettes		Number of active rosettes		Number of zymcompl. rosette	
	in 1 µl	in 1 $\mu$ l	%	in 1 µl	%	in 1 µl	%
Tumor bearing patients $n = 22$	$1376.8 \\ \pm 72.23$	$\begin{array}{c} 644.20 \\ \pm 44.00 \end{array}$	$\begin{array}{c} 46.40 \\ \pm 2.42 \end{array}$	$314.61 \\ \pm 28.54$	$\begin{array}{c} 22.15 \\ \pm 2.20 \end{array}$	$381.3 \\ \pm 46.78$	$\begin{array}{c} 28.51 \\ \pm 3.0 \end{array}$
Normal control $n = 26$	$\begin{array}{c}1539.0\\\pm63.0\end{array}$	$914.0 \\ \pm 36.0$	${}^{60.69}_{\pm 1.07}$	$341.3 \pm 24.4$	$22.9 \pm 1.54$	$293.5 \pm 17.0$	$\begin{array}{c} 20.11 \\ \pm 0.69 \end{array}$
Significance (Student t)	no	p < 0.01	p < 0.01	no	no	no	p < 0.05

Data are the average  $\pm$  standard error.

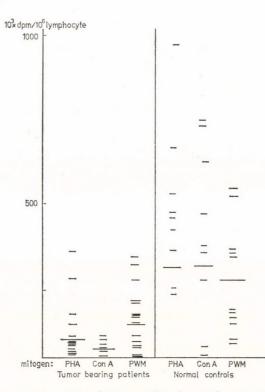


FIG. 1. Lymphocyte transformation in tumour bearing patients and healthy blood donors

tions. WILCOXON's test showed a significant difference at the 95% level between tumour-bearing patients, healthy control persons and patients without malignancy.

#### TABLE II

PHA transformation in 11 patients operated upon with malignant tumour before and 6 weeks after surgery

	A	Average of 10 <sup>6</sup> lymphocyte in DPM $\pm$ s.e.						
		in pooled serum	tested in autologous serum					
Before operation	56.787	$\pm 15.596$	41.373	$\pm 15.496$				
After operation	77.628	$\pm 22.197$	27.659	$\pm$ 7.869				

11 patients of this group were tested six weeks after operation. In these cases the lymphocyte transformation test was done also with lymphocytes with 20% inactivated patient's plasma in the culture medium in pre- and post-operative cultures. In both cases parallel cultures were set up with the usual 20% normal pooled inactivated AB serum (Table II). With samples taken 6 weeks after operation Student's t test showed a significant difference at the 95% level between average stimulation rates of cultures containing the patients' own versus pooled AB plasma. The slight difference seen in the pre-operative cultures became significant six weeks after operation.

In Table II average values are shown. It is seen that the patients' plasma did not always inhibit lymphocyte stimulation; in three cases it even enhanced the reaction.

As regards to the suppressive serum factor of the tumour-bearing patients, when five such sera were pooled the inhibitory effect was the same as with the strongest single one (Fig. 2).

#### Killer cell activity

Results by ADCC were significantly lower (p < 0.05) with the patient's (Fig. 3B) than with the healthy donor's lymphocytes (Fig. 3A) in all four target-effector cell ratios.

## Discussion

In recent years numerous methods have been developed to study malignant processes and their immunological background. They may have some prognostic value and reflect the progression of disease or the effect of therapy [10, 43].

T and B cell ratio was extensively studied in cancer patients. T cell depression was observed in most patients with visceral carcinoma but normal levels were found in early cancer of the breast, sarcoma and melanoma patients [4, 32, 38, 42, 43, 45].

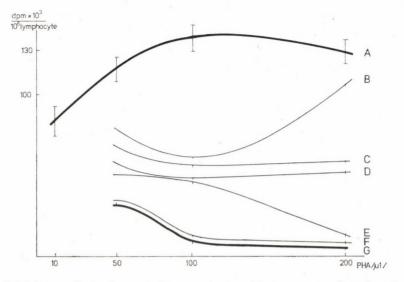


FIG. 2. Inhibitory effect of serum from patients with tumour on lymphocyte transformation in healthy blood donors. A) Lymphocyte transformation in a healthy blood donor; B—F) Sera five tumour-bearing patients tested on normal lymphocytes; G) Mixed sera of the five patients tested on the same normal lymphocyte

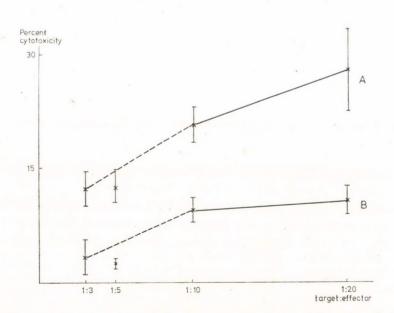


FIG. 3. Killer activity of healthy blood donors and patients with tumour before the operation in ADCC system

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The relation to prognosis of delayed hypersensitivity tests was tested with both DNCB and recall antigens [43]. Tuberculin sensitivity was examined in a recent study of melanoma patients showing a good response in early cases and a poor one in patients with metastases [34].

The relevance of lymphocyte transformation to non-specific mitogens in malignancy has also been established. In lung carcinoma a good correlation was found between diminished response to PHA and patient survival [15]. PHA reactivity generally weakens in severely ill patients [9, 12, 13, 45] and especially in cases with rapid progression [14], while in the early stage the response may be the same as in healthy persons [8].

For assessing immune competence, MIKULSKI et al. [27] recommended testing of the cytotoxicity in vitro with chromium release in tumour patients. Nowadays, apart from testing for tumour specific reactivity, which will not be discussed here, assays for ADCC [22] and natural killer activity [28] are growing in significance.

Increasing attention is payed to macrophage and granulocyte function, phagocytosis and or chemotaxis being more and more involved in the testing of immune function [21, 43].

Early changes in humoral immunity are less common than the impairment of cellular immune function. Direct assays of humoral immunity are generally less informative. The antibody titres against ubiquitous bacteria may be depressed, and the antibody induction time may be delayed [23] in cancer patients. Hypocomplementemia observed in 10% of sera of patients with malignancies [39] the elevated level of circulating immune complexes is also characteristic [36]. Alterations of the immunoglobulin levels are characteristic of certain tumours, for example IgM-depletion in malignant melanoma [37], or elevated IgG levels in carcinoma of the breast and prostate [1, 35].

More interesting and more important are the assays for blocking serum factors. After the first observations [14, 18] it was suggested that the in vitro inhibiting effect of the patients' plasma represents the enhancement phenomenon [6, 27]. Recently, UEDA [40] emphasized the prognostic value of serial examinations of the immunosuppressive effect of patients' plasma in ovarian cancer patients.

Our early results with serum inhibition [29] were consistent with other data. Though we could not make further progress in the study of malignancy, we were able to show a relation between the inhibiting effect of pregnant women's sera and a low-molecule soluble mediator isolated from the serum donors' lymphocytes [25, 26].

In the present report we summarized our experience with cellular immune competence testing in patients with malignancy before surgery and partly after surgery.

With the different rosette techniques our results were generally in accordance with other data: E-rosette values were reduced in patients with cancer of the oesophagus, cardia, or the stomach. The active rosette count was not significantly different from the average of the control groups, but, as shown in Table I, with a wide scatter. The mainly B-lymphocyte-detecting zymosancomplement rosette counts were significantly lower if the percentages were compared but not significantly when expressed in absolute numbers. RAND et al. [33] obtained similar results and to explain the discrepancy they supposed that in cancer patients cell surface receptors are blocked by serum factors, mainly immune complexes. As during progression of the malignant disease more and more soluble immune complexes are detectable [8, 17], this probably applied to our cases, too.

Lymphocyte transformation tests were done with three different mitogens: ConA stimulating the T lymphocytes, PHA mainly the T lymphocytes, and PWM the B lymphocytes through a helper T cell effect. Significant differences were found between cancer patients and control cases with all three mitogens (Fig. 1). Here each patient and control case is represented by the individual maximum response. It is interesting, how little variation occurred in the patient group.

In 11 patients lymphocyte transformation was repeated six weeks after the operation. In all these cases the effect of autologous plasma was also studied. Table II shows these results. As already mentioned, the main tendency was inhibition, but in some cases the patient's plasma enhanced the reaction. The inhibiting effect became significant only after surgery, apparently because six weeks after the operation the basic values (with pooled AB plasma) were much higher than before.

Studying the plasma inhibition effect, five such sera were mixed and tested again (Fig. 2), with the result that the strongest effect was not influenced by diluting the sera with each other. This is in accordance with our recent unpublished observation: to demonstrate the inhibitory effect it is not necessary, to add the autologous serum previously to the culture medium. Minute amounts of patient serum added to the usual AB serum containing cultures, were sufficient to produce the inhibitory effect.

Killer cell activity by ADCC was significantly lower in the patients' than in healthy donors' lymphocytes in all four target-effector cell ratios. With the view in mind that K cells have an important role in tumour rejection [7] it is interesting that only in six patients did the difference prove highly significant. The fact that the findings of LANG et al. [22] were controversial, might be explained by the longer history and previous treatment of most of their patients. Our patients all untreated received no previous treatment, were diagnosed recently before surgery, so probably most of them were in the stage when K cell activity was going to be exhausted.

The present observations do not allow definite prognostic calculations. They suggest to follow by multiple testing many patients as possible after surgery and perhaps also during chemotherapy.

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# Zelluläre Immunfunktion beim Menschen im Falle eines im oberen Teil des gastrointestinalen Trakts sitzenden Krebses

#### Von

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Bei 30 Patienten wurde die zelluläre Immunfunktion der Lymphozyten 1 Tag vor der Operation untersucht. In 11 Fällen wurde die Untersuchung 5 Wochen nach der Operation wiederholt und die ermittelten Ergebnisse den Werten von 26 gesundenen Blutspendern gegenübergestellt. Es ergab sich, daß die E-Rosettenwerte in der Gruppe der Patienten signifikant niedriger waren. Die Rosettenzahl des Zymosankomplement erwies sich — insofern das prozentuelle Verhältnis verglichen wurde — für signifikant niedriger, während sich beim Vergleich der absoluten Zahlen keine Signifikanz meldete. Mit Hilfe des Lymphozyten-Transformationstests, bei Anwendung aller 3 Mitogene (PHA, ConA, PWM) zwischen Patienten und Kontrollen eine signifikante Differenz nachgewiesen werden. In 11 Fällen wurde anläßlich der postoperativen Kontrolle auch der Effekt des autologen Plasmas in den Kulturen untersucht. Die Haupttendenz war die Inhibition, in einigen Fällen wurde aber die Reaktion durch das Plasma des Patienten intensiviert. Die Aktivität der Killer-Zellen war bei den Patienten signifikant niedriger als bei den Zellquotienten der verschiedenen Target-Effektoren der Lymphozyten der normalen Blutspender.

## Клеточная иммунная функция у пациентов с раком верхних отделов желудочно кишечного тракта

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Исследовали лимфоциты у 30 пациентов за день до операции для определения клеточной иммунной функции. У П пациентов исследования повторили спустя шесть недель после операции. Способность к Е-розеткообразованию у пациентов была очень сильно понижена по сравнению с таковой в контрольной группе (26 здоровых доноров). Число зимозан-комплементных розеток было достоверно (статистически значимо) ниже при сравнении процентов, но не было достоверным, когда сравнивали абсолютные значения. Выявлена 7остоверная разница в интенсивности трансформации лимфоцитов, стимулированных митогенами (ФГА, КонА, ПВМ). У II пациентов, которым сделали повторные тесты после операции, исследовали также действие аутологичной плазмы на клеточные культуры. Главной тенденцией было ингибирование, но в некоторых случаях плазма пациентов усиливала эту реакцию. Антителозависимая цитолитическая активность была ниже у лимфоцитов пациентов по сравнению с лимфоцитами здоровых доноров в случае разных клетокмишений.

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