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In vitro and Clinical Characterisation of a Newcastle Disease Virus-modified Autologous Tumour Cell Vaccine for Treatment of Colorectal Cancer Patients

Winfrid Liebrich, Peter Schlag, Maria Manasterski, Burkhard Lehner, Michael Stöhr, Peter Möller and Volker Schirrmacher

A virus-modified autologous tumour cell vaccine prepared from human colorectal cancer cells is described. After dissociation an average of 5×10^7 cells/g tissue were obtained from primary tumours and 9×10^7/g tissue from metastases with an average viability of 72% and 51%, respectively. Following irradiation (200 Gy), inactivation of the proliferative activity of the cells was demonstrated by their degeneration in tissue culture and the absence of incorporation of ^{3}H-labelled thymidine. One third of the cells were still metabolically active, as shown by the incorporation of ^{3}H-uridine and a mixture of ^{3}H-aminoacids. The dissociated cells expressed MHC class I and II antigens in a qualitatively similar way to tissue sections. Epithelium-specific antigens (detected by MAb HEA125) were expressed on an average of more than 75% cells of the suspension, while leucocyte-specific antigens (detected by MAb CD53) were expressed on an average of less than 25% cells. The vaccine was prepared by admixing the nonlytic strain Ulster of Newcastle disease virus (NDV) with the tumour cell suspension. The NDV adsorption at tumour cells was shown by electron microscopy. Clinically, the treatment with the vaccine was associated with an increased sensibilisation against autologous tumour cells, measured by DTH skin reactivity. First results in 23 patients with colorectal liver metastases who underwent "curative" liver resection followed by vaccination show a clear correlation between the induced increase of DTH skin reaction against autologous tumour cells and the recurrence-free interval. No correlation was found for DTH reaction caused by standard antigens (Mérieux test), NDV alone or autologous normal liver tissue. The results demonstrate the possibility of preparing immunogenic virus-modified autologous tumour cell vaccine from colorectal cancer tissue, which could be used for cancer therapy.
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INTRODUCTION

MODIFYING TUMOUR cells by means of viruses may increase their immunogeneity [1, 2]. The commonly used procedures in clinical tumour vaccination studies is the use of oncolysates or membranes from tumour cells infected with viruses such as vaccinia virus [3] parainfluenza virus [4], or Newcastle disease

virus (NDV) [4].

Experimental work revealed that in many tumour systems the immunogenicity of vaccines of intact tumour cells was higher than that of cellular material like membranes [5–7]. These findings are also true for the ESb lymphoma model of the mouse: a vaccine was prepared from intact tumour cells infected with NDV, and successfully used for postoperative immunotherapy of micrometastases [8–12].

Despite curative tumour resection in a large number of human tumours, the prognosis is affected by micrometastases already existing at the time of surgery. The concept of active specific immunotherapy (ASI) seems to be promising in the treatment of microscopic tumour foci. Based on our preclinical investigations [8, 9, 11, 12] we designed a similar protocol for the preparation of an autologous human tumour cell vaccine. This vaccine consists of irradiated intact tumour cells whose cell surface was modified by NDV infection.

Clarification was required of (1) whether a sufficient amount of appropriate tumour cells can be isolated from surgically removed solid tumours or metastases, (2) whether cells lose some of several surface antigens during the preparation procedure, (3) whether such isolated cells can be modified by adsorption to NDV, and (4) whether it is possible to induce an immunological response (e.g. DTH reaction) in patients, when using the material as a vaccine.

In this article we describe our experience in preparing this autologous NDV-modified tumour cell vaccine for colorectal carcinomas and present preliminary clinical results.

MATERIALS AND METHODS

Tissue preparation and dissociation

To characterise the "vaccine", a variety of tissue specimens of primary colorectal cancer and its liver metastases were extensively investigated. Tumour and normal tissue was collected under sterile conditions. Representative tumour specimens were taken from the peripheral part of the mass. The mucosa was removed in a distance of at least 5 cm from the tumour edges. It showed no morphological changes under the light microscope. Normal liver was taken in a distance of about 5 cm from the tumour.

The tissue was rinsed using Hanks' balanced salt solution (HBSS) supplemented with 100 IE/ml penicillin, 100 μg/ml streptomycin and 0.05 mg/ml gentamycin, dissected and treated with collagenase and DNase (200 U/ml, respectively 500 Kunitz U/ml; both type I, Sigma). The number and viability of the cells were determined by trypan blue exclusion. Afterwards cells were stored in liquid nitrogen [13, 14].

Modification of the tumour cells using NDV

The avirulent strain Ulster of NDV (kindly provided by Dr P.J. Russel, Royal Veterinary College, London) was propagated and isolated [11, 15]. To adsorb the virus, 1×10^7 cells were thawed, washed with HBSS, and irradiated with 200 Gy (^{132}Cs source, Gammacell 1000, Atomic Energy of Canada, Ottawa, Ontario). In analogy with the procedure worked out in the ESb animal tumour model [11] and *in vitro* tests [9], the cell material (10^7 cells) was mixed afterwards with 32 HAU of NDV [16] and incubated at 37°C for 1 h.

Immunoperoxidase staining

Cytospins were made of 2×10^5 tumour cells (7 min, 55 *g*). Immunoperoxidase staining was carried out [17]. The following mouse monoclonal antibodies were used: HEA125 (directed against the epithelium-specific antigen Egp34 [18]), CD53 (reactive with all bone marrow derived cells; unpublished, kindly provided by Dr Moldenhauer, Heidelberg), and W6/32 (anti-MHC class I [19]), 2.06 (anti-MHC class II [20]).

Transmission electron microscopy

The virus-infected cells were fixed first with 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.2 (C-buffer; dimethylarsinic acid sodium salt·$3H_20$). After washing, the specimens were postfixed with 1% osmium tetroxide in C-buffer. Following stepwise dehydration in acetone, the samples were embedded in epoxy resin according to Spurr [21]. Thin sections were stained with uranyl acetate (1% in 70% acetone) following lead citrate solution (1%, aqueous).

Flow cytometric analysis

Determination of cell viability. Flow cytometry was carried out with 5×10^6 cells each, dissociated from eight individual colorectal tumour tissues, before and after irradiation with 200 Gy. The cell samples were incubated in Hoechst-33662 staining solution (enters living and dead cells), and a propidium iodide solution (enters only dead cells) was added prior to flow analysis as described in detail previously [22].

Analysis of cell surface antigens with the two monoclonal antibodies HEA125 and CD53. 5×10^6 dissociated cells of two primary tumours and two liver metastases were separately investigated. Cells were incubated with the first antibody for 30 min, incubation with a FITC-labelled goat anti-mouse antibody (Jackson, Baltimore) followed for additional 30 min. Thereafter cells were counterstained with propidium iodide (PI) for exclusion of dead cells (PI-positive). FITC analysis was restricted to viable cells (PI-negative).

Incorporation of tritium-labelled thymidine, uridine and a mixture of aminoacids

5×10^5 cells each of 25 preparations (14 colorectal primary tumours and 11 liver metastases) were incubated with 37 kBq/ml ^{3}H-thymidine (specific activity: 777 MBq/mg; Amersham), ^{3}H-uridine (specific activity: 759 MBq/mg; Amersham) or a ^{3}H-aminoacids mixture (leucine, specific activity: 35.7 GBq/mg; lysine monohydrochloride, specific activity: 15.5 GBq/mg; phenylalanine, specific activity: 27.6 GBq/mg; proline, specific activity: 31.23 GBq/mg; tyrosine, specific activity: 20.7 GBq/mg; Amersham) in HBSS at 37°C for 30 min. The samples were precipitated in trichloroacetic acid, washed with ethanol and air dried. Following the addition of scintillation solution, the activity of the specimens was determined using a beta counter (LS 7000, Beckmann) [23].

Lethally irradiated cells in tissue culture

In three cases irradiated cells (200 Gy) dissociated from primary tumours were cultivated in RPMI supplemented with

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Table 1. Characteristics of the patients undergoing ASI following resection of colorectal liver metastases (n = 23)

	No. of patients
Age, years (range)	61 (30–70)
Sex (f/m)	1/1.3
Synchronous metastases	8
Metachronous metastases (total)	15
Interval	
< 12 months	4
12–36 months	9
>36 months	2
Type of liver resection	
Hemihepatectomy	9
Liver segment resection	6
Plurisegmentectomies	8

10% fetal calf serum and antibiotics (100 IE/ml penicillin and 100 μg/ml streptomycin). Viability and metabolic activity were tested prior to, immediately after, and 1, 2 and up to 10 days after irradiation, using the trypan blue exclusion test and incorporation of tritium labelled thymidine, uridine and aminoacids (specific activities above).

Patients

To test the efficacy of the vaccine, patients with radical resection of colorectal liver metastases without clinical and histological evidence of residual cancer were vaccinated. No additional postoperative chemotherapy or radiotherapy was given. Patients gave informed consent for this study in accordance with institutional and federal guidelines.

The first 23 vaccinated patients with a follow-up of at least 12 months were evaluated. Patient characteristics are depicted in Table 1. Routine follow-up examinations including liver ultrasound and computed tomography (CT), chest X-ray and carcinoembryonic antigen monitoring were performed at 3 month intervals.

Vaccination protocol and DTH reactivity testing

Vaccination was started 2–3 weeks after liver resection. The treatment schedule included five vaccinations at 14 day intervals (Table 2). At the same time as the first vaccination the patient's *a priori* sensitivity against their autologous tumour cells was measured. For this purpose 1×10^7 tumour cells not treated with NDV was injected. The same challenge test was done after the last inoculation to test the patient's reactivity after the vaccination procedure. 3 months later a sixth vaccination and a third challenge test were done to test for long-lasting immunity. For specificity control, NDV and cells from normal autologous liver tissue were injected.

Table 2. Vaccination protocol

Days after liver resection	14	28	42	56	70	160
Procedure no.	I	II	III	IV	V	VI
10^7 tumour cells + 32 HAU NDV (= vaccine)	*	*	*	*	—	*
10^7 tumour cells (challenge)	*	—	—	—	*	*
10^7 liver cells (control test)	*	—	—	—	*	*
32 HAU NDV (control test)	*	*	*	*	—	*
Mérieux test (standard antigens)	*	—	—	—	*	*

* = injection, — = no injection.

Table 3. Comparison between cells dissociated from tissue of primary tumours or liver metastases of colorectal cancer patients

	Cells	
Parameters	Primary tumour	Liver metastases
Viability (trypan blue exclusion test; n = 19)		
After dissociation	72 (12)%	51 (22)%
After storage in liquid N_2	75 (22%)	52 (22)%
Viability (n = 8)		
Before irradiation	82 (15)%	ND
After irradiation	84 (9)%	ND
Yield of cells (n = 19)		
Per g tissue	$5\ (3) \times 10^7$	$9\ (2) \times 10^7$
Total	$9\ (6) \times 10^7$	$18\ (9) \times 10^7$
Incorporation of uridine or aminoacids		
>500 cpm and trypan blue viability	14/27	3/29
>75% after irradiation (200 Gy)	samples	samples
Incorporation of uridine or aminoacids		
<500 cpm and trypan blue viability	7/27	4/29
<75% after irradiation (200 Gy)	samples	samples

Mean (S.D.)
ND = not determined.

To test the patient's sensibility to standard recall antigens, a commercial skin test (Mérieux Multitest, Institute Mérieux, Norderstedt) was used. This consisted of an emulsified antigen solution to tetanus diphtheria, streptococci, tuberculin, *Candida*, trichophyton, and *Proteus* [34].

A DTH skin reaction was assessed 24 h after injection. Only local induration was measured. An induration less than 3 mm in diameter was recorded as negative.

Sensitisation against autologous tumour cells during ASI was assumed, when the difference between the recorded DTH of the first and sixth vaccinations was ≥3 mm. For the commercial skin test the sum of all indurations greater than 2 mm in diameter was divided by the number of positive reactions.

The results of the DTH analyses were correlated with *in vitro* parameters (cell viability) and clinical outcome (recurrence free survival). Statistical evaluation was performed using Pearson's correlation test.

RESULTS

Yield of cells and viability

After enzymatic treatment an average (S.D.) yield of $5\ (3) \times 10^7$ cells/g tissue was obtained from colorectal carcinoma and of $9\ (2) \times 10^7$ cells/g from metastatic tissue. About 72% or 51% of the dissociated cells, respectively, were viable trypan blue excluding cells (Table 3). This relatively high yield of cells was achieved only after one step of enzymatic treatment with an incubation time of 30 min.

Cytological characteristics of the cell suspensions

More than 75% of the cells were HEA125 positive (epithelium-specific monoclonal antibody; Fig. 1b, Table 4), whereas less

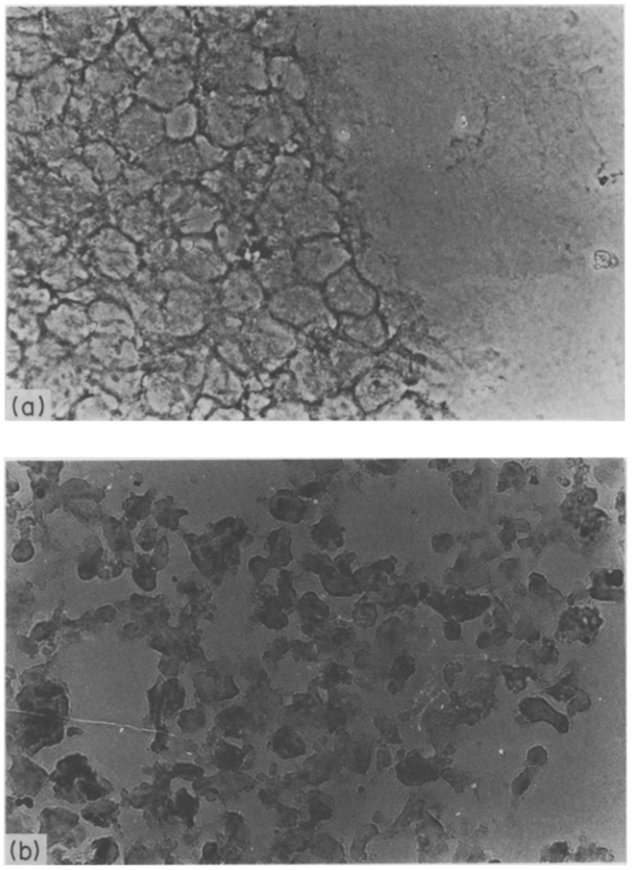

Fig. 1. Immunohistology of frozen tissue sections and isolated cells. (a) Tissue section. Distribution of the antigen detected by the monoclonal antibody HEA125. Left = stained membranes of cells of the tumour area; right = negative normal mucosa tissue. (Phase contrast optics, ×700.) (b) Dissociated cells. Cells and cell membranes stained by the monoclonal antibody HEA125. (Phase contrast optics, ×400.)

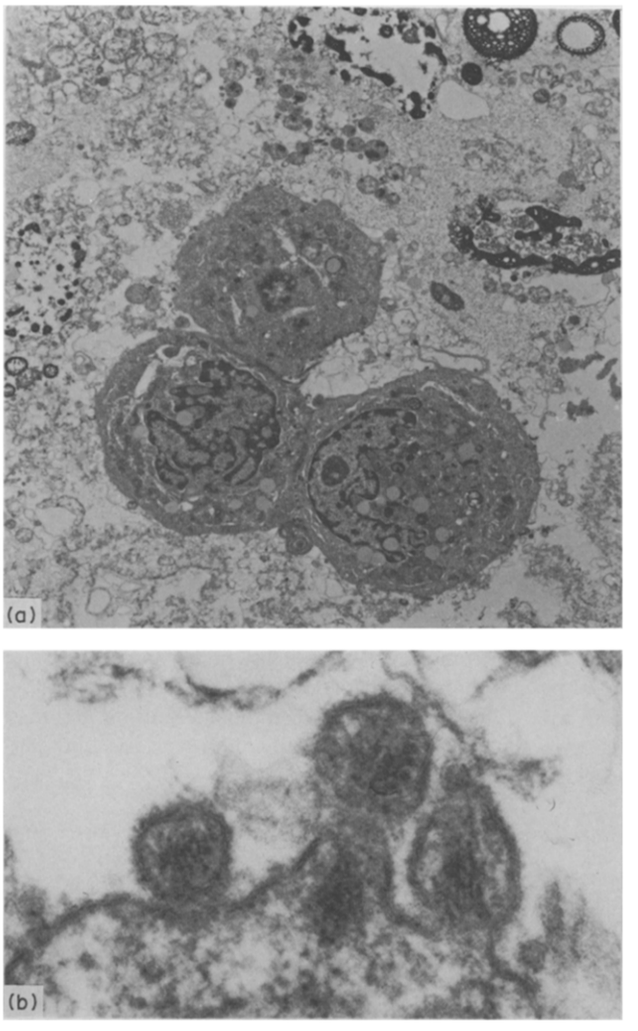

Fig. 2. Transmission electron microscopy of a vaccine sample. (a) Beside intact cells, membrane fragments and degenerating nuclei are visible (×3300.) (b) Newcastle disease virus adhering to a cell membrane (×100 000).

than 25% were CD53 positive (bone marrow derived cells; Table 4).

The data were confirmed for the CD53 analysis using flow cytometry of cells dissociated from tissue of two primary tumours and two liver metastases (Fig. 3c). Data obtained from HEA125 receptor analysis, however, were not supported by flow cytometry: only 20–50% of the viable cells analysed were HEA125 positive (Fig. 3b).

Beside intact cells the dissociated material could sometimes contain cell debris depending on the individual consistency of the tumour tissue. Preparations from liver metastases often contained up to 50% cell debris. Using transmission electron microscopy it was identified as cell fragments, membrane vesicles, mitochondria, and degenerating nuclei (Fig. 2a).

Table 4. Staining distribution of the monoclonal antibodies HEA125 (epithelial), W6/32 (MHC I), 2.06 (MHC II), CD53 (lymphoid) in 20 preparations of isolated colon cancer cells

Stained cells	HEA125	W6/32	2.06	CD53
0%	0/20	0/17	0/17	6/17
≤10%	0/20	0/17	9/17	7/17
20–40%	0/20	0/17	4/17	4/17
40–80%	0/20	0/17	4/17	0/17
>80%	20/20	17/17	0/17	0/17

Adsorption of NDV to dissociated carcinoma cells

Electron microscopic investigations of cells incubated for 1 h with NDV showed that in one of seven to ten cell sections virus particles were identified at the cell surface of epithelial cells (Fig. 2b). NDV particles were additionally adsorbed to fragments of cell membranes or were present as aggregates between the cells.

Influence of enzymatic treatment and irradiation on the expression of cell surface antigens

The original tissues, the cells after dissociation, after irradiation, and after irradiation and NDV incubation were investigated by means of the monoclonal antibodies HEA125, CD53, W6/32, and 2.06 for the presence of corresponding antigens (Table 4).

In tumour tissues HEA125 stained the area of the tumour and epithelial cells but not stroma or infiltrating lymphocytes (Fig. 1a); CD53 stained lymphoid but not epithelial cells. In cell suspensions prepared from individual tumours or from metastatic tissues there was no difference in the staining distri-

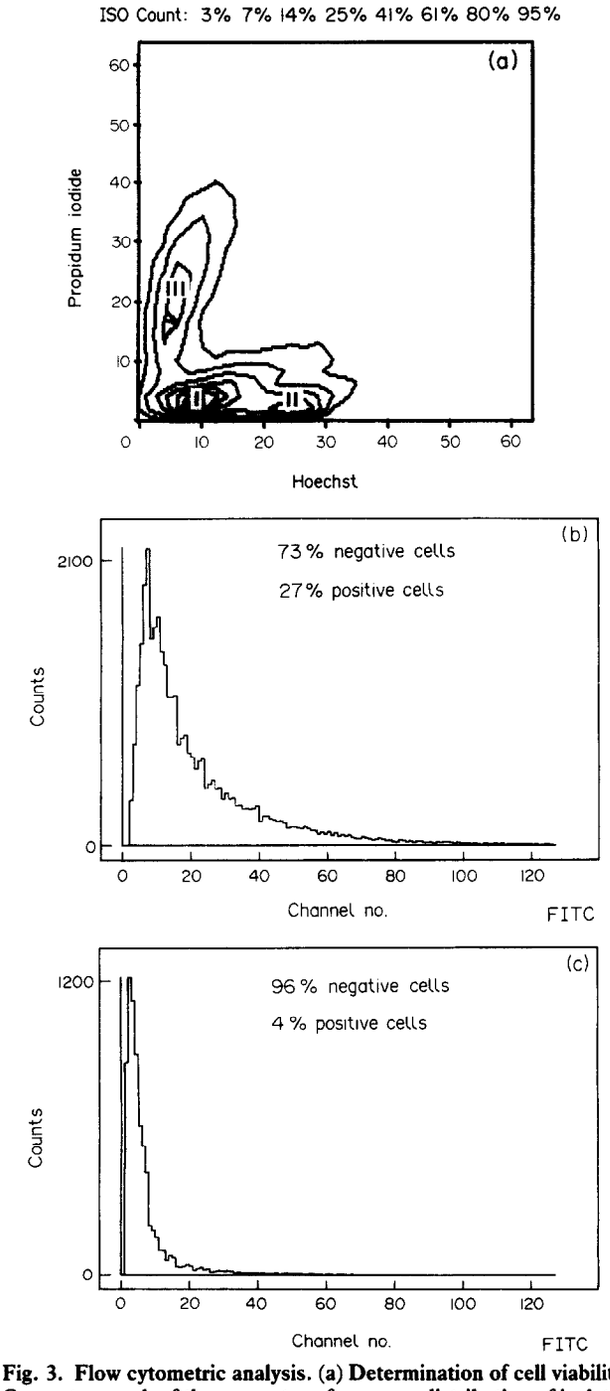

Fig. 3. Flow cytometric analysis. (a) Determination of cell viability. Computer graph of the percentage frequency distribution of isolated colon-cancer cells after double-staining with Hoechst-33662 (=Hoechst) and propidum iodide (=PI). Clusters I and II indicate viable cells (Hoechst stained) cluster III represents dead cells (PI stained). Sample of cells before irradiation. (b) and (c) Analysis of surface receptors of living cells of a randomly chosen preparation of dissociated colon-cancer cells. The FITC-positive cells are at the right of channel 20. (b) Computer analysis of the percentage distribution of HEA125-positive cells, n = 34492. (c) Computer analysis of CD53-positive cells, n = 7155.

bution of the four antibodies at the cell surface during the various steps of vaccine preparation. An example of staining of HEA125 at the surface of cells after irradiation and NDV incubation is shown in Fig. 1b.

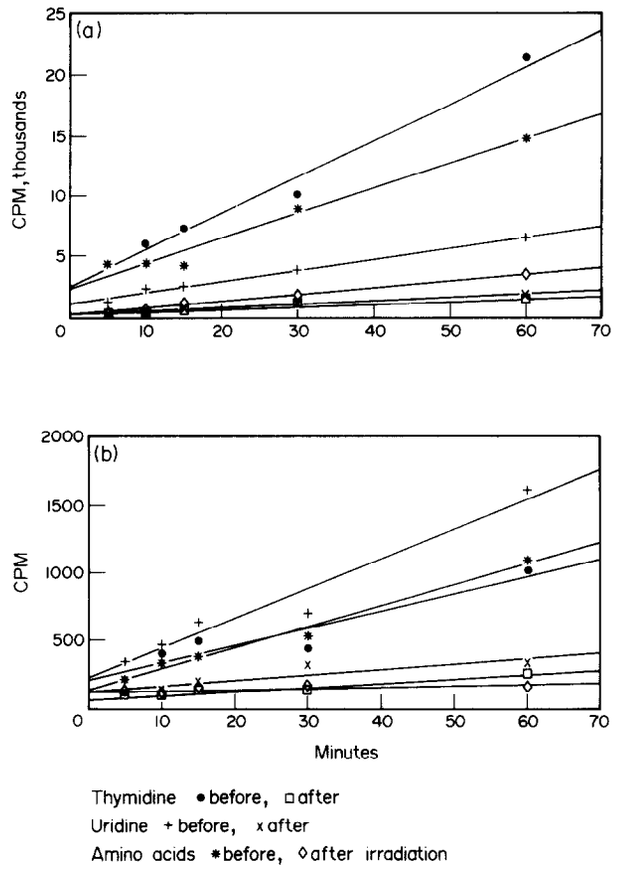

Fig. 4. Incorporation kinetics of tritium-labelled thymidine, uridine and a mixture of aminoacids into 5 × 10^5 colon cancer cells before (upper lines) and after (lower lines) irradiation of cells with 200 Gy. (a) The established colon carcinoma cell line HT 29. (b) A sample of dissociated human colon carcinoma cells.

Influence of irradiation on survival of the isolated cells in tissue culture, viability, DNA, RNA, and protein synthesis

Survival in tisue culture. The viability (trypan blue exclusion) of dissociated cells decreased to 20% within 3 days after irradiation with 200 Gy. The incorporation of tritium-labelled thymidine, uridine and a mixture of aminoacids was inhibited already after the first day in culture.

Viability. The computer graph of the flow cytometric analyses shows three different clusters. The clusters I and II in Fig. 3a represent the Hoechst-33662 stain (= living cells in G_0 [I] or in S and M phase [II]), cluster III represents propidium iodide (= dead cells) stained cells (Fig. 4a). There is no difference in the mean [S.D.] percentage distribution of the clusters before irradiation (I: 69 [13]%; II: 13 [1]%; III: 18 [15]%) and after irradiation (I: 71 [16]%; II: 13 [9]%; III: 16 [15]%).

Biological activity. Figure 4 displays the kinetics of incorporation of radioactive precursors of DNA, RNA and protein synthesis in cells of an established carcinoma line HT 29 (a) and in cells dissociated from a primary carcinoma (b). Cells of the (control experiment) tumour line incorporated about 10 times more of the tritium-labelled thymidine, uridine or aminoacids than a randomly chosen preparation of isolated colon tumour cells.

In samples of dissociated primary tumours and metastases containing cells with ^{3}H-thymidine incorporation exceeding 500

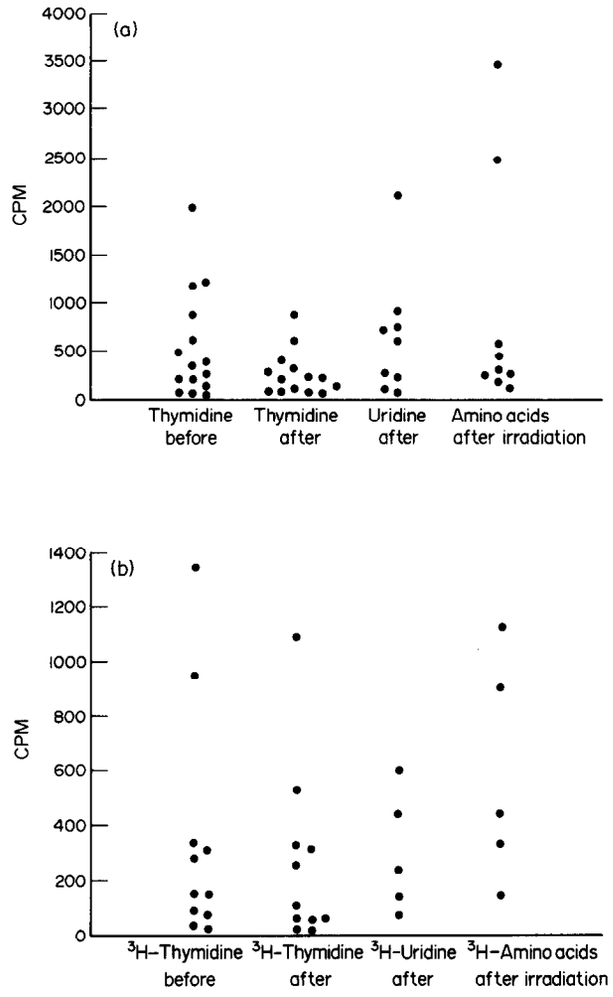

Fig. 5. Incorporation of tritium-labelled thymidine before and after irradiation and uridine and a mixture of aminoacids after irradiation with 200 Gy. (a) Incorporation into isolated colon cancer cells. (b) Incorporation into isolated cells of liver metastases.

cpm/5 × 10^5 cells before irradiation (7/25), no incorporation was seen after irradiation. In 6 of 20 specimens ^{3}H-uridine and ^{3}H-aminoacid incorporation exceeded 500 cpm/5 × 10^5 cells after irradiation (Fig. 5a).

In cells dissociated from liver metastases, the data of viability determination by trypan blue exclusion did not correlate with those of incorporation of tritium-labelled uridine or aminoacids (Fig. 5b). In cells dissociated from primary tumours, however, some correlation was detected: in 14 of 27 cases the viability was 75% or higher and the incorporation of ^{3}H-uridine or ^{3}H-aminoacids exceeded 500 cpm/5 × 10^5 cells (Table 3).

Preliminary clinical results. The vaccine was tolerated well by all 23 patients immunised. Beside mild fever on the day of the vaccination no severe side-effects were observed during an observation period of 12 months. In 13 patients an increase in their reactivity against the vaccine was observed during the vaccination cycle. 11 patients had a relevant increased reactivity against autologous tumour cells (>3 mm) at the fifth or sixth vaccination compared to prevaccination status (challenge test). In a follow-up of at least 12 months, 12/23 patients developed tumour recurrence. The recurrence-free interval was strongly correlated to the increase of the DHT reactivity against autologous tumour cells (Pearson's correlation coefficient = 0.7895; Fig. 7a). No correlation could be observed comparing DTH against the standard antigens of the Mérieux multitest with the enhancement of the DTH reaction against applied 10^7 autologous tumour cells (correlation coefficient = 0.2139; Fig. 7b). There was no correlation between the increase of the DTH against standard antigens of the Mérieux test and recurrence free interval (correlation coefficient = 0.0930; Fig. 7c) Sensitisation against normal liver and NDV could be excluded: there were no changes in the DTH reaction against normal liver and NDV alone in the course of ASI. In addition, no correlation was observed between DTH against tumour cells and DTH against 10^7 cells of normal liver (correlation coefficient = 0.6702; Fig. 7d) or 32 HAU NDV alone (correlation coefficient = 0.1600; Fig. 7e). Surprisingly, a negative correlation appeared in outlines at that early point of time comparing recurrence-free interval and viability of cells used in the vaccine (correlation coefficient = −0.6481; Fig. 7f).

DISCUSSION

The average yield of cells isolated from primary tumours was higher than previously described [13, 30]. The percentage of tumour cells in our dissociated cell preparations was at least 75% as determined by immunohistochemistry, and less than 50% as determined by flow cytometry. This discrepancy is explained by the fact that only living cells were included in the flow cytometric analysis.

Hoechst-33662 stain penetrates into cells and accumulates in the chromatin of the cell nucleus, causing a fluorescence intensity, which is proportional to the DNA content of the cells [22]. The flow cytometric data revealed cells with different DNA content. According to the amount of DNA, the cells of cluster II could either be polyploid tumour cells, cells in S-phase, or mitotic cells (M phase). Other authors found that even after low dose irradiation most cells degenerated following incubation in tissue culture due to chromosomal and DNA damage [34, 26]. From our *in vitro* culture and incorporating studies obtained after 200 Gy irradiation we conclude that the dissociated, irradiated cells did not replicate anymore and, therefore, could be used as a safe vaccine of intact tumour cells.

Our results, obtained with surface-labelling antibodies, revealed that the surface characteristics of the cells were not negatively affected during the various necessary steps of preparation. This also gives promise for using these isolated tumour cells as a vaccine.

The efficacy of the postoperative therapeutic effect of vaccination in the animal tumour model depended on the viability of inoculated cells and on successful NDV adsorption [9, 10, 11, 15]. Metabolic activities of cells dissociated from liver metastases did not correlate with the cell viabilities as determined by trypan blue exclusion. Preparations with trypan blue incorporating cells could still be metabolically active. Cells dissociated from primary tumours exhibited a better viability and metabolic activity.

The adsorption of NDV to cells dissociated from tissue of colorectal primary tumours and liver metastases was shown by electron microscopy. The results suggest that NDV is absorbed to the cell surface. The immunological advantages of such NDV modified vaccines have previously been reviewed in detail [8, 9, 12, 27].

Our studies demonstrate that despite identical experimental procedures for dissociation, cell suspensions from different patients showed variations in cell yield, viability and metabolic activities. The individual materials thus differ from the homo-

geneous tissue culture cells used for vaccines in animal models [9]. In addition, it has to be noted that none of the dissociated human tumours had such a high viability or rate of incorporation of tritium-labelled thymidine, uridine, or aminoacids as cells of an established human colon carcinoma cell line (HT 29). Even in cells of primary tumours characterised by a high viability (trypan blue) and metabolic activity (incorporation >500 cpm/5 × 10^5 cells) the incorporation rate was only about 10% of that seen by the HT 29 cells or the ESb mouse lymphoma cell line. On the other hand our data are in good agreement wih results obtained for other human malignancies [23].

A further problem, which is negligible when using a cell line, is the high percentage of additional cell debris. Concomitant administration of debris might negatively affect the therapeutic results. On the other hand, a possible positive effect due to adjuvant stimulation of the immune system has been discussed [28, 29].

Compared to vaccines prepared with BCG (e.g. [13, 30]) our vaccine was tolerated without severe side-effects. In 11/23 of the vaccinated patients a relevant increase of the DTH reaction against autologous tumour cells was observed. DTH reaction as a monitoring parameter has so far been a matter of controversy. Some authors found a close correlation between DTH response and *in vitro* tests [2, 31, 32], while others reported the opposite [5, 25, 28, 30]. Taking into account that in our experience no correlation between DTH reactivity to standard antigens (Mérieux test) and DTH response to autologous tumour cells was found, we conclude that the increase in sensitivity against the autologous tumour cells is related to the vaccination performed. In addition, our results confirm a correlation between recurrence-free interval and the increase of the tumour-cell DTH. Therefore, the data suggest that DTH is helpful in monitoring active specific immunotherapy.

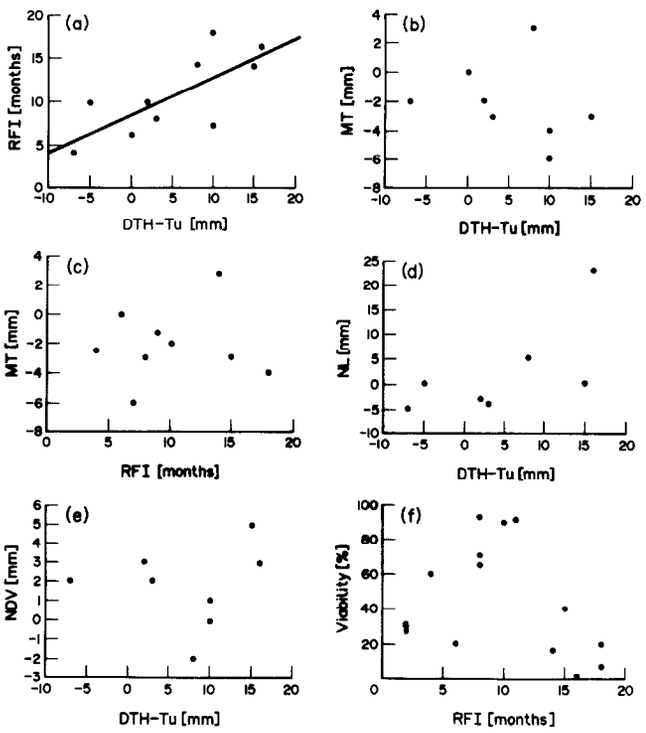

Fig. 7. Correlation of clinical and experimental characteristics of the vaccine. (a) Correlation of the recurrence free interval with the incidence of DTH reactivity to 10^7 autologous tumour cells (challenge test). (b) Correlation of the DTH caused by the standard antigens of the Mérieux test and the rise of DTH against autologous tumour cells. (c) Correlation of the DTH caused by the standard antigens of the Mérieux test and the recurrence free interval. (d) Correlation of DTH of 10^7 cell of autologous normal liver with the rise of DTH against 10^7 autologous tumour cells. (e) Correlation of DTH against 32 HAU NDV alone and the increase of the DTH reaction to autologous tumour cells. (f) Correlation of recurrence free interval and viability of the cells used for vaccination.

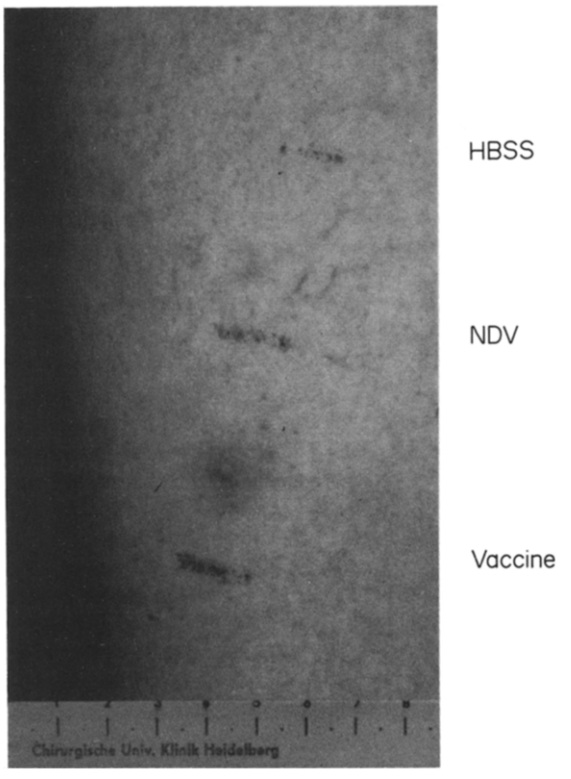

Fig. 6. DTH skin response 24 h postinoculation of the vaccine. HBSS = Hanks' balanced salt solution (control 1), NDV = 32 HAU Newcastle disease virus (control 2) and vaccine = 1 × 10^7 isolated tumour cells modified by 32 HAU NDV.

The recurrence-free survival was negatively correlated to the viability of the cells used for the vaccine. This may be due to the fact that incorporation studies did not correlate with the results of the trypan blue exclusion test. Because of the lack of tumour material, however, incorporation studies could not be performed in parallel for the 23 patients undergoing the standardised vaccination protocol. Further studies will be needed to clarify this observation.

In summary, it is possible to prepare an autologous tumour cell vaccine from human colorectal carcinomas (primary tumours as well as metastases) and to modify the immunogenicity of the cells by adsorption to NDV. The vaccine is non-tumorigenic but retains its antigenic characteristics. In terms of viability and metabolic activity cells of the vaccine differ from those of established cancer cell lines [9, 10, 11, 15]. Using the vaccine clinically, sensibilisation against autologous tumour cells was induced when measured by the challenge test at the fifth or sixth vaccination. Preliminary clinical results demonstrate a strong correlation between recurrence-free interval and the amount of increase of DTH reactivity against autologous tumour cells following vaccination. Future randomised studies will show whether the described treatment improve patients' prognosis.

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