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Presence of normal human cell surface antigens in plasma of athymic mice bearing a human colon carcinoma and in normal human plasma

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Summary. The mixed haemadsorption (MHA) method was employed for detection of several normal antigenic components on the surface of human colon carcinoma cells (HT-29). The antigens were expressed by cells in monolayer cultures and in suspensions prepared by monolayer trypsinization, and by cells of tumours growing progressively in athymic mice. The plasma of such animals bearing medium sized and large, non-necrotic tumours contained all the antigens, as determined by the radial diffusion immune haemolysis method (RDIH); the plasma of animals with small or large heavily necrotic tumours did not contain detectable amounts of any of the determinants. The half-life of the determinants in the circulation as extracellular entities was ca. 20 h. The same antigens, and fibronectin, were found to be ubiquitously represented in normal human plasma. It is proposed that the presence of membrane antigens in plasma is the result of physiological shedding of cell surface constituents by living cells.

Introduction

We have reported previously [22, 23, 24] that various normal and neoplastic cells growing in tissue culture shed macromolecular components from the surface as a normal consequence of membrane turnover. We now posed the question whether physiological cell surface shedding also takes place in vivo. To this effect, we examined the plasma or sera of athymic (nude) mice bearing tumours of human colon carcinoma cells (HT-29 cell line) for the presence of five surface antigens, employing the radial diffusion immune haemolysis (RDIH) assay developed by us [24]. We chose this model system in light of the consideration that human neoplasms developing in athymic mice generally retain their original histophysiological properties [10, 25], and of our earlier findings that HT-29 cells express on their surface, and shed, appreciable quantities of β_2 microglobulin (β_2M), histocompatibility (HLA) antigens and blood group substance A under in vitro growth conditions [23, 24]. In the present communication, we show that these as well as another surface antigen studied are present in the circulation of athymic mice bearing progressively growing, non-necrotic HT-29 implants, and that the half-life of the determinants in the circulation is of short duration ($t_{1/2} = \sim 20$ h). Because the substances studied are normal constituents of many human cells, we also examined their presence in normal human plasma (or sera); all the determinants were indeed found to be ubiquitious components of human blood. The RDIH test was chosen for several reasons. Its level of sensitivity is similar to that of radioimmunoassay; it holds the advantages of easy performance and rapidity; it does not require radioactive reagents; and as described by Molinaro et al. [18], it can be applied to study antigen release by single cells.

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Materials and methods

Cells and cultures. Cells of the HT-29 human colon adenocarcinoma cell line (kindly provided by Dr. J. Fogh, Sloan Kettering – Memorial Cancer Center, NY) were grown as monolayers in 9 cm tissue culture plates (Nunc, Roz Kilde, Denmark), employing Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, N.Y., USA) supplemented with 15% fetal calf serum (Gibco) and antibiotics. Incubation was at 37° C in a 95% air – 5% CO_2 humidified atmosphere. Cells for sub-culturing of the line (passage monolayers) were obtained by treating the monolayers with a trypsin-EDTA solution: 50 mg trypsin (1 :250; Difco, Detroit Michigan, (USA) and 200 mg EDTA (BDH Analar, Poole, England) dissolved in 1 l phosphate buffered saline (PBS) containing 2.42 gm Tris buffer (Fisher, Fair Lawn, N.J. USA), pH 7.4.

Cell suspensions. Suspensions of HT-29 cells were prepared by treating monolayer cultures or tumour fragments with the trypsin-EDTA solution for 20 min at 37 °C. The cells were then washed in culture medium, centrifuged at 50 g for 10 min, and resuspended in medium. Cell viability was ascertained by 0.1% trypan blue exclusion.

Athymic mice. The animals were young adult females of the BALB/c nu/nu strain raised in the colony of Hebrew University-Hadassah Medical School. Breeder animals are kept under strict isolation conditions in laminar flow hoods. The animals were maintained on a diet of irradiated Purina Chow and acidified (pH 2.8) autoclaved drinking water.

Tumours. HT-29 cells (5×10^6) in 0.2 ml saline suspensions prepared from monolayer passage cultures were injected SC into the right outer thigh. Palpable tumours appeared in most animals within 4 weeks and grew progressively. Tumour volumes were ascertained at sacrifice of the hosts

or after surgical removal, under anaesthesia, of the growths. The tumours were grouped according to volume into three categories: small (ca. 0.3 cm^3), medium (ca. 3 cm^3), and large (ca. 7 cm^3). Some of the large growths were heavily necrotic.

Mouse and human plasma and sera. Mice were bled from the inner canthus of the eye using capillary tubes; the plasma or sera derived were stored at −90 °C. Human plasma or sera were obtained from (presumably) healthy donors working in our laboratories and from the blood bank of Hadassah Hospital, Mount Scopus (courtesy of Dr. R. Sharon); they were stored at −20 °C. For fibronectin (FN) determinations, only fresh human plasma was utilized. Of the 50 human sera and plasma tested 3 showed non-specific haemolysis in the assay and were excluded from compilation of the results.

Antibodies. The following antibody preparations were employed for coating eryhrocytes in the mixed haemadsorption (MHA) and RDIH tests:

Rabbit anti-human β_2 M, monospecific IgG fraction (Dakopatts Reagents Ltd., Copenhagen, Denmark). This antiserum did not react with mouse β_2 M in serum, as determined by radioimmunoassay employing the Phadebasmicro test radioimmunoassay RIA kit (Pharmacia Diagnostics).

Anti-HLA serum, obtained from a multiparous polytransfused woman (kindly supplied by Dr. C. Brautbar of our laboratories); the antiserum was tested with a panel of lymphocytes from 60 donors, and found to be reactive against a broad spectrum of HLA-A, B, and C antigens.

Anti-blood group substances A and B prepared from human blood (Gamma Biological, Inc., Houston, Texas, USA).

Monoclonal antibody F_4, prepared according to the method of Köhler and Milstein [16] by immunization of BALB/c mice with intact, living HT-29 cells in suspension. The antibodies produced by the hybridoma were harvested from ascitic fluid, and tested by indirect immunofluorescence against a broad panel of different types of cells in culture, both normal and neoplastic. F_4 was reactive against a surface antigen expressed by all human cells tested, and non-reactive for cells of other species (mouse, rat, dog, chicken). The target antigen, which we designate human species specific surface antigen (HSSA), is a 17×10^3 dalton protein, as determined by immunoprecipitation and sodium dodecylsulphate-polyacrylamide gel electrophoresis.

Rabbit anti-FN was prepared in our laboratories. Chromotographically pure human plasma FN, made as described by Vuento and Vaheri [30], was the immunizing antigen. The globulin fraction of the antiserum was absorbed by passage through sepharose bead columns of human plasma depleted of FN. Monospecificity of the absorbed globulin fraction was indicated by immunoelectrophoresis against human plasma: Only one precipitation line was seen, identical with that obtained against purified FN.

Coating of erythrocytes with antibody. The chromium chloride method was employed, as detailed by Plesser et al. [24]. For coating of erythrocytes (sheep red blood cells, SRBC), the following dilutions in saline of the antibody preparations were employed: anti-β_2M, 1:5; anti-HLA, 1:10; anti-blood group substances A and B, 1:20; F_4 1:1; anti-FN, 1:20. As controls in all test runs performed, erythrocytes were coated with normal rabbit serum, pre-immune rabbit serum, irrelevant rabbit or human antisera, or irrelevant mouse monoclonal antibodies.

Mixed haemadsorption assay (MHA)

On cell monolayers. HT-29 cell monolayers in 3.5 cm culture plates (Nunclone, Roz Kilde, Denmark) were washed once with DMEM. Then 2 ml of a 0.4% suspension in DMEM of SRBC coated with antibody was added to the monolayers. After standing for 1 h at room temperature, the plates were rinsed gently at least four times with PBS; after such repeated washing, there was no retention of SRBC coated with any of the control sera or antibodies.

On cells in suspension. To a 5 µl pellet of mildly centrifuged HT-29 cells was added 1 ml of a 0.4% suspension in DMEM of SRBC coated with antibody. The mixture was agitated by mild vortexing, and left to stand for 2 h at room temperature. It was then again subjected to mild vortexing, and samples removed for microscopic examination. The SRBC coated with control sera or antibodies exhibited no binding to the cells.

On frozen tumour sections. Drops of suspensions of SRBC coated with antibody were layered on cryostat sections. After 60 min at room temperature, the slides were gently washed with PBS until no control-coated SRBC remained bound to the sections. The slides were then fixed with 2.5% glutaraldehyde and stained with haematoxylin-eosin.

The intensity of the haemadsorption reaction was estimated microscopically, and expressed in the following units: 5 = large numbers of SRBC attached to all or most of the cells (rosette formation so strong as to mask most of the target cells); 4 = large numbers of SRBC attached to ca. 75% of the cells (prominent rosette formation); 3 = moderate numbers of SRBC attached to ca. 50% of the cells; 2 = several SRBC attached to ca. 25%–35% of the cells; 1 = a very few SRBC attached to less than 25% of the cells; 0 = no discernible attachment of SRBC to target cells.

Radial diffusion immune haemolysis (RDIH) in gel assay. In this method, the solution to be tested for the presence of antigen is introduced into wells punched into a layer of agarose-containing antibody-coated erythrocytes. Diffusion of this solution into the gel brings the antigen into contact with the erythrocytes. Upon flooding the plates with more of the same antibody and complement, a zone of haemolysis forms around each well, proportional to the concentration of antigen in the test fluid. The method has been described in detail by Plesser et al. [24]. The following modifications of the method were employed here: The amount of agarose-coated erythrocyte mixture introduced into the plates (5 cm, Nunclone) was 1.5 ml. The plates were pre-warmed to 45 °C. The mixture was shaken in the plates to assure even distribution, and permitted to solidify at room temperature on a perfectly horizontal surface. To prevent formation of a miniscus in the plates, the rims were coated with paraffin before introduction of the mixture, thus leading to formation of an even sheet of gel entirely parallel with the bottom of the plate. Between 6 and 24 wells were punched into the gel of each plate with a

3-mm bore needle, and the plates covered and permitted to stand for 30 min before introduction of serum or culture medium into the wells, as described previously [23]. The plates were flooded with additional antibody and complement after the test solution had diffused out of the wells. Haemolysis was complete after 1 to 4 h, depending on the antigen and antibody combination in each system. The plates can be preserved for later reading of the haemolytic zones by covering with 2.5% glutaraldehyde in PBS and storage at 4 °C. The gel removed from the plates can be dried on filter paper by the usual procedure used for polyacrylamide gels [13], and stored indefinitely.

Reliability of the RDIH method for detection of antigens in serum. In order to ascertain the reliability of the RDIH method for detecting antigens in serum, we conducted a preliminary test comparing RDIH and RIA for quantification of one determinant, β_2M. The values obtained by the two methods were virtually identical for four randomly chosen human sera tested (1.72 ± 0.34 µg/ml for RIA, 1.79 ± 0.27 for RDIH).

Results

Experiments with athymic mice

Inoculation into athymic mice of living HT-29 cells of suspensions obtained by monolayer trypsinization led to the development of well encapsulated adenocarcinomas in most of the animals. Palpable masses were evident within 4 weeks after inoculation and grew progressively, reaching volumes of ca. 7 cm^3 within 12 weeks. The surface antigens examined were strongly expressed on cells of the passage monolayers, of the suspensions employed for inoculation, of suspensions prepared by trypsinization of tumours of all sizes (although apparently to a lesser extent on cells of small tumours), and of monolayers established from the tumour suspensions (Fig. 1). Figure 2 depicts the typical appearance of the MHA reaction for one of the antigens (β_2M) on HT-29 cells derived from a large tumour, in suspension and in monolayer culture. The MHA reactivity was also pronounced for the determinants on frozen sections of the tumours. Figure 3 illustrates the reaction for β_2M on a section of a tumour with necrotic areas. Whereas the neoplastic tissue is highly positive, the host connective tissue strands and the areas of necrosis are negative.

Analysis of the plasma of tumour bearing mice for the presence of the four antigens was conducted using the RDIH assay. The results are summarized in Table 1. The plasma of animals with large and medium size tumours were invariably positive for all determinants, as were the conditioned media of the in vitro monolayer cultures. The plasma of control animals not inoculated with HT-29 cells were wholly negative. None of the antigens could be detected in any plasma taken from animals with small tumours or with large, highly necrotic growths.

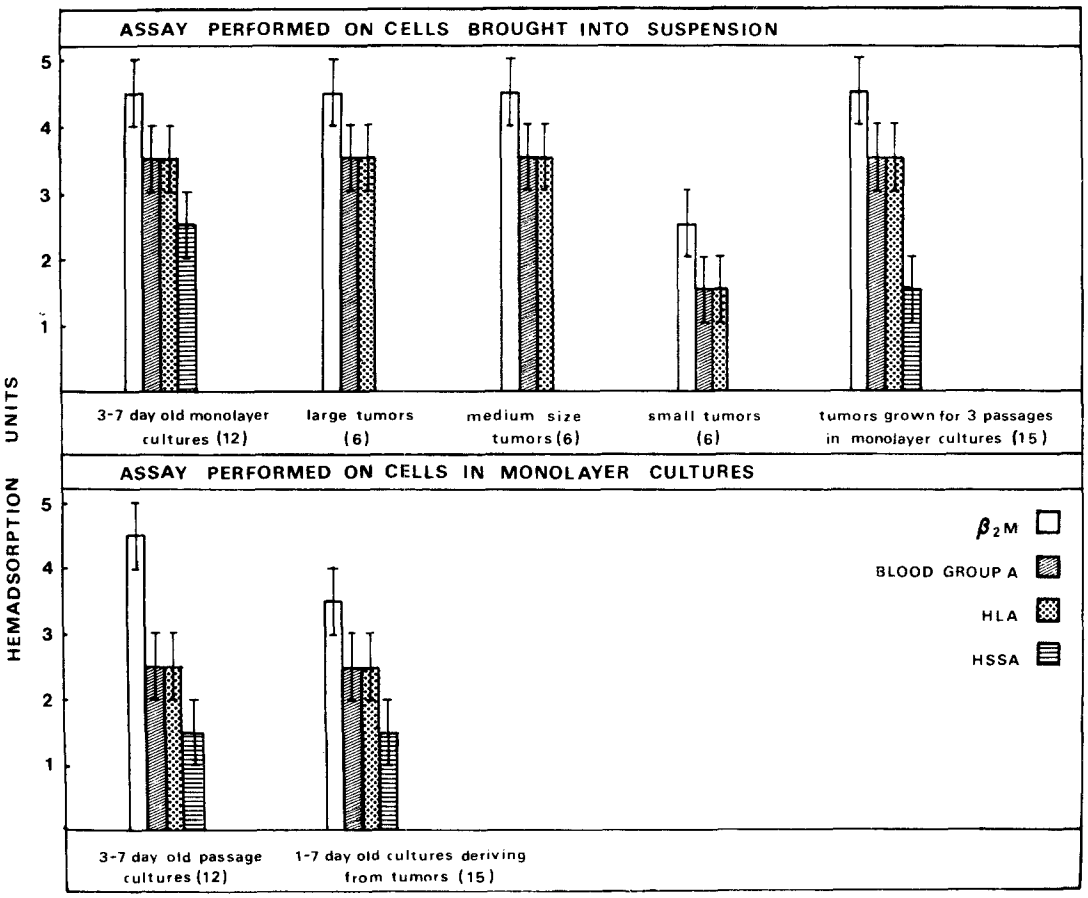

Fig. 1. Expression of four cell surface antigens on human colon carcinoma cells (HT-29) as detected by mixed haemadsorption. The numbers in parentheses indicate the numbers of tests performed, in triplicate, on cells of each group. The values shown represent the range of reaction intensities (see *Materials and methods*) for each group. No differences were found in reaction intensities between cells of monolayers cultured for 1 to 7 days

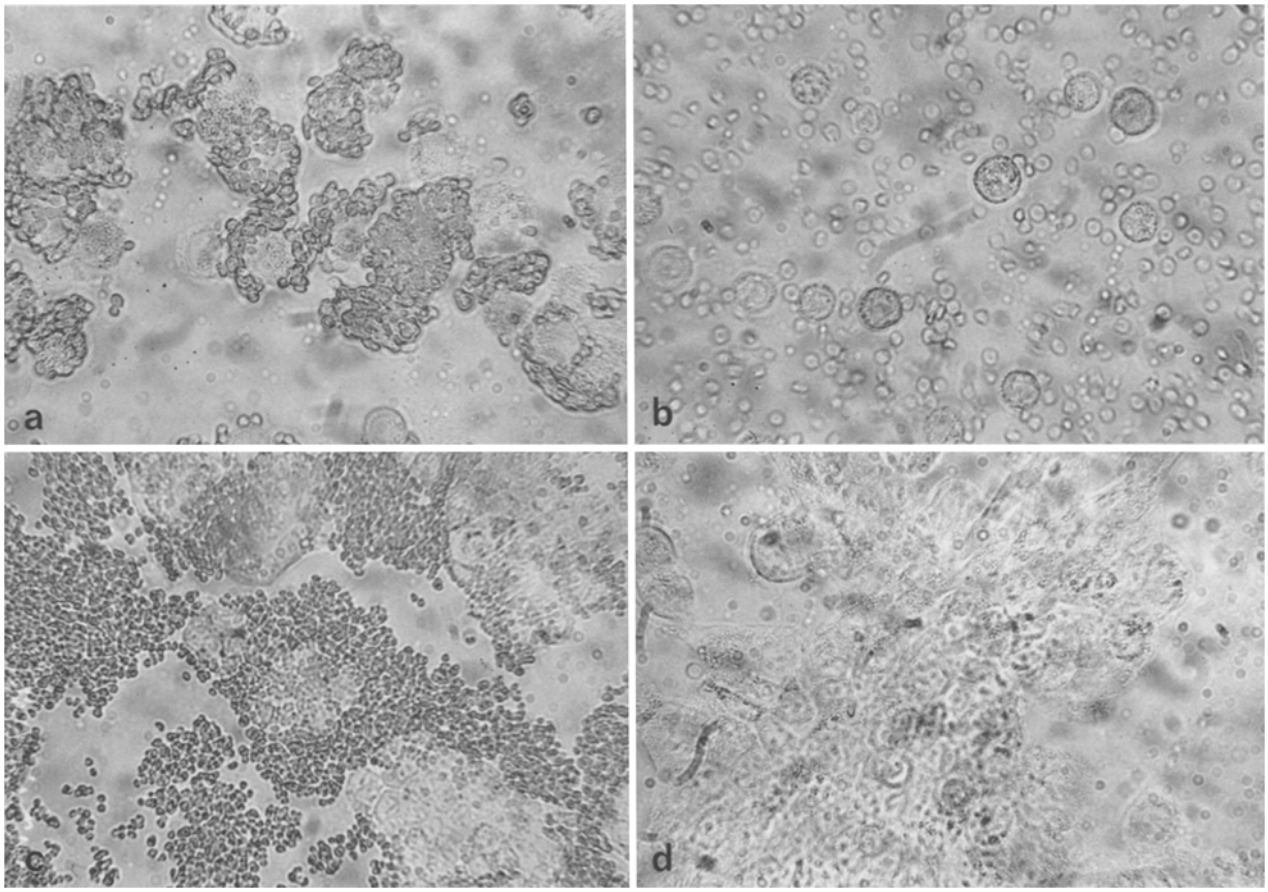

Fig. 2 a–d. MHA reaction for β_2 microglobulin on HT-29 cells derived from a large tumour in an athymic mouse. **a** Reaction of cells in suspension with SRBC coated with anti-β_2M antibodies. **b** Reaction of cells in suspension with SRBC coated with normal rabbit serum; there is no adherence of erythrocytes to the tumour cells. **c** Reaction of cells in monolayer culture with SRBC coated with anti-β_2M antibodies. **d** Reaction of cells in monolayer culture with SRBC coated with normal rabbit serum; eryhtrocytes are not seen. (**a** and **b** $\times 400$) (**c** and **d** $\times 250$)

In order to ascertain the life span of the shed antigenic entities in the circulation, we tested the plasma of mice with large non-necrotic tumours before and at intervals after surgical removal of the growths. As seen from the results depicted in Fig. 4, all four antigens disappeared within hours after removal of the tumour, whereas the plasma antigen concentration in mice with similarly large tumours that were not extirpated remained constant. It is noted from the slope of the regression lines that the rate of elimination was similar for all the determinants.

Experiments with human plasma and sera

If the appearance of cell surface antigens in the circulation is indeed a consequence of the normal, ongoing process of surface membrane turnover and shedding by both normal and neoplastic cells, normal human blood would be expected to contain such determinants. We tested 45 normal human plasma and sera for presence of the same antigens studied in the circulation of athymic mice bearing HT-29 tumours and of two additional determinants – FN and blood group substance B. Our findings are presented in Tables 2 and 3.

All sera proved positive for β_2M HLA, HSSA and FN. Because pure preparations of β_2M and FN were available for the construction of standard curves, absolute values could be obtained for these two substances. The values found by us, 1.92 μg β_2M/ml and 311 μg FN/ml, accord with those reported in the literature, 0.8–4.2 μg β_2M/ml and 330 μg FN/ml.

The results for blood group antigenic activity are shown in Table 3. Of 16 sera from A individuals and 14 from B donors, all were positive for the respective blood group and negative for the other. Of 13 AB sera tested, 3 were positive for blood group substance A only, 4 for blood group substance B only, and 6 for both. Of 12 0 sera examined, 9 were negative for both antigens and 3 were, surprisingly, positive for both.

Discussion

The observations described here indicate the pronounced constancy of expression of several antigens examined on the surface of HT-29 cells. The determinants could be detected on cells passaged in monolayers and on cells brought into suspension by monolayer trypsinization, and on cells of tumours developing in athymic mice and on cells of such tumours brought into suspension and cultured.

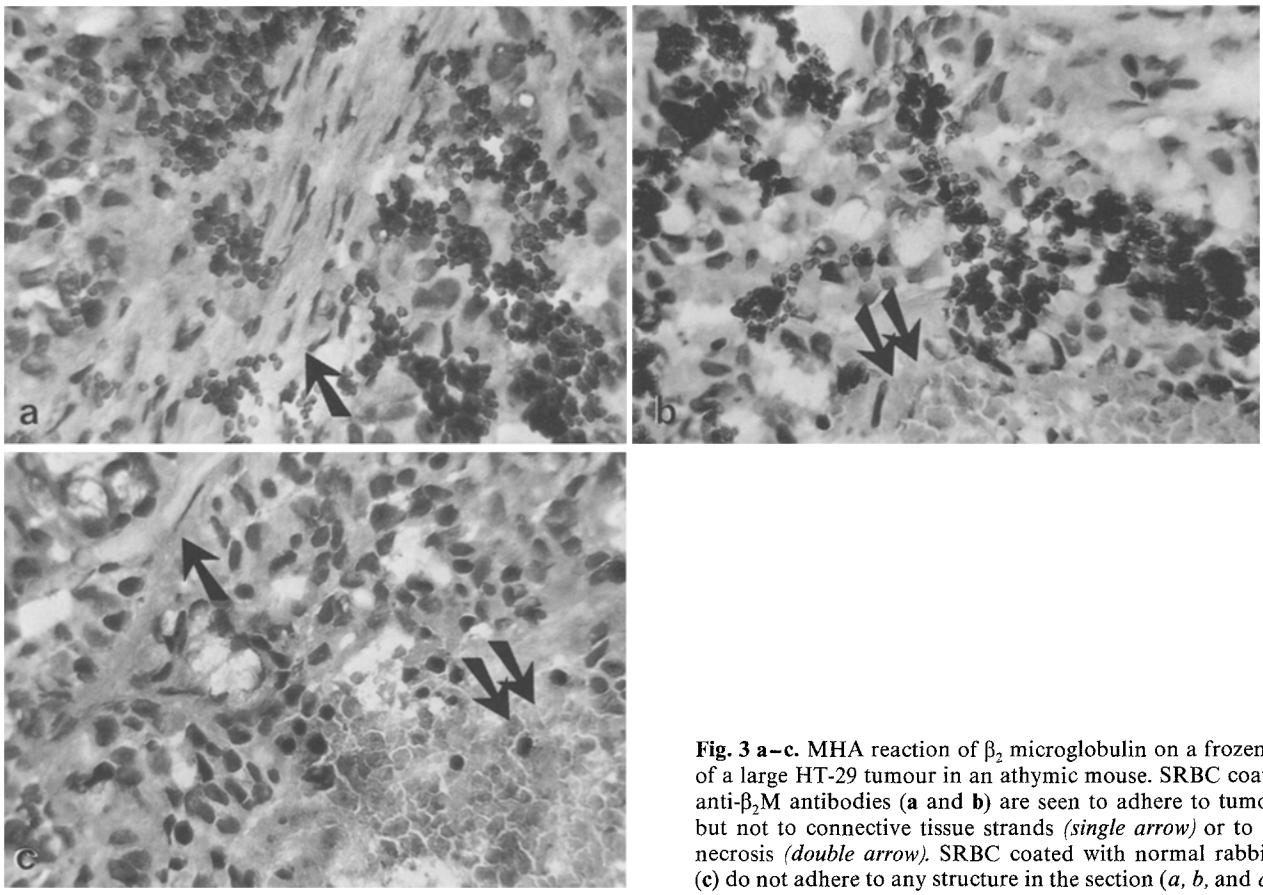

Fig. 3 a–c. MHA reaction of β_2 microglobulin on a frozen section of a large HT-29 tumour in an athymic mouse. SRBC coated with anti-β_2M antibodies (**a** and **b**) are seen to adhere to tumour cells but not to connective tissue strands *(single arrow)* or to areas of necrosis *(double arrow)*. SRBC coated with normal rabbit serum (**c**) do not adhere to any structure in the section (*a*, *b*, and *c* × 400)

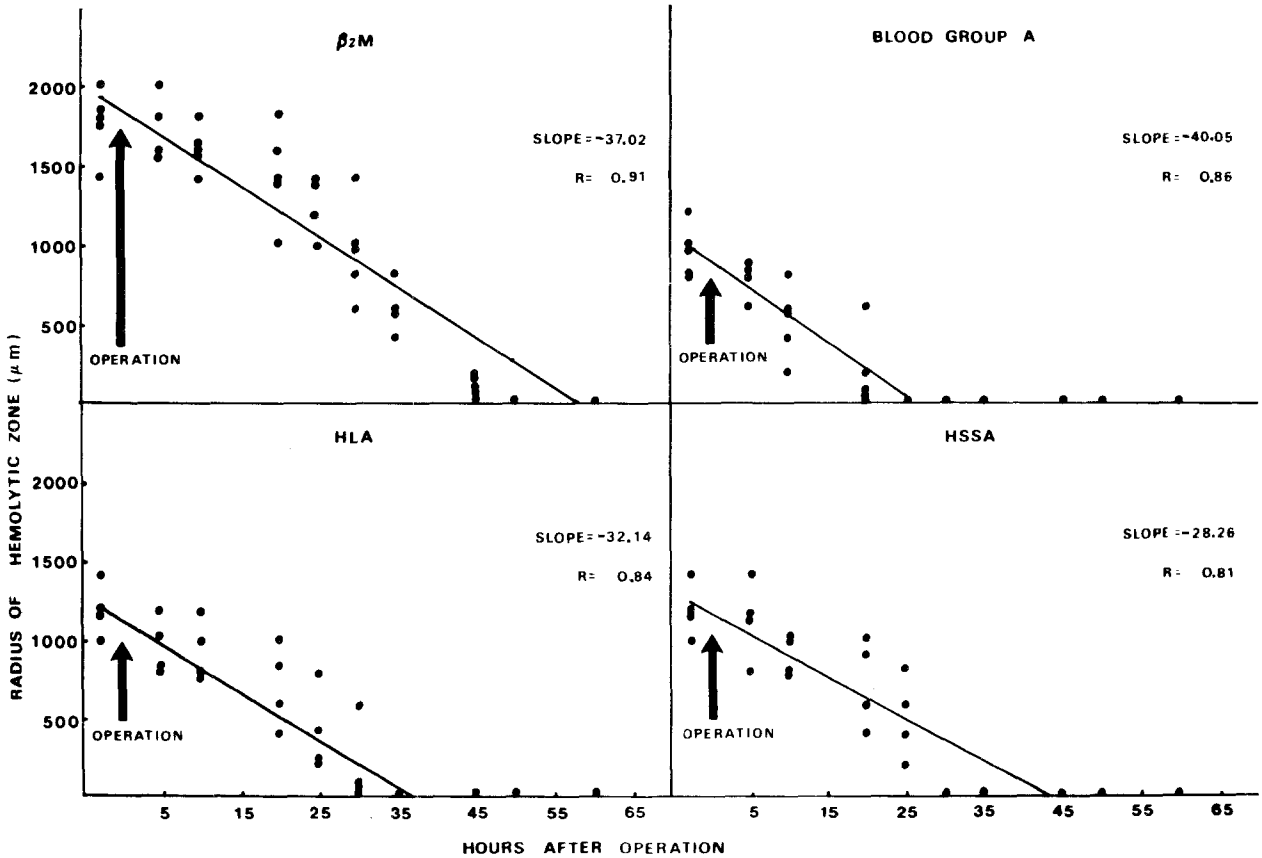

Fig. 4. Rate of disappearance of human cell surface antigens from the curculation of nude mice after surgical removal of a large human colon carcinoma implant. In tumour bearing mice not subjected to surgery, the level of antigens did not change over the time period examined. Regression lines were determined by the least-squares method

Table 1. Presence of human cell surface antigens in plasma of athymic mice bearing human colon carcinoma tumours as detected by RDIH

	Antibodies			
	Anti β_2M	Anti blood group substance A	Anti HLA	Monoclonal antibody F_4
	Radius of haemolytic zone (μm) [a]			
Plasma of animals with large tumours	1700–2150 (10)	1400–1950 (6)	1950–2250 (10)	1050–1500 (5)
Plasma of animals with large necrotic tumours	0 (7)	0 (4)	0 (7)	ND
Plasma of animals with medium sized tumours	1450–2000 (10)	1000–1650 (6)	1500–2000 (10)	850–1200 (5)
Plasma of animals with small tumours	0 (10)	0 (6)	0 (10)	0 (5)
Plasma of animals not inoculated with tumour cells (controls)	0 (10)	0 (6)	0 (10)	0 (5)
Media from 3–7 day old HT-29 passage cultures	2250–2700 (12)	1650–1800 (10)	1700–2050 (10)	950–1250 (4)
Media from 1–4 day old cultures of HT-29 cells derived from tumours in athymic mice	2150–2700 (16)	1700–1850 (10)	1950–2450 (19)	ND

[a] Each group consisted of 3–4 animals. Plasma was tested individually and repeatedly, in triplicate each time. The numbers in parentheses indicate the numbers of tests performed on plasma from each group of animals. The values shown represent the range obtained for each group of plasma. Culture media taken on different days after seeding was also tested individually and repeatedly, in triplicate; the numbers in parentheses indicate the numbers of culture media tested, and the values the range obtained for each group of media. ND – not done. It is noted that parallel testing of sera and plasma always yielded the same results; the data for sera and plasma are accordingly pooled, and presented under the heading of "plasma"

The plasma of athymic mice bearing medium sized and large non-necrotic tumours contained all these antigens, whereas the plasma of animals bearing small or large, necrotic tumours lacked all determinants in amounts discernible by the RDIH method. Representation in plasma of cell surface components could accrue either from cell death and disintegration, or from shedding from the cells as part of the process of surface membrane turnover. We have adduced evidence previously that the presence of surface antigens in tissue culture media is, in fact, a consequence of physiological shedding and not of cell destruction [22, 24]. The present observation that the surface antigens surveyed are detectable in the plasma of hosts carrying sizeable, non-necrotic tumours but not in those of animals with heavily necrotic growths argues in favour of the manifestation of physiological shedding in vivo as well as in vitro.

Failure to detect the antigens in the plasma of mice with small tumours is likely to be due to release of only limited amounts, below the threshhold of detectability. Also, their short life span as extracellular entities in the circulation would prevent their accumulation to the point of assessability.

The shedding of surface components by tumour cells has been described by many investigators [3, 4, 5, 7, 11, 12, 14, 15, 17, 20, 26, 27]. Most of these studies were conducted with the aim of ascertaining the implications of the phenomenon for early and differential diagnosis of neoplastic disease, therapeutic monitoring and immuno-imaging. The suggestion has also been advanced that the level of shedding correlates with the degree of malignancy of tumour cells in vivo [5]. The present study emphasizes that shedding of *normal* cell constituents is part of the physiological process of surface membrane turnover by *both* neoplastic and normal cells.

The surface components of HT-29 cells studied here are expressed by a variety of normal human cells. Our finding that normal human plasma contains all these (and other) normal membrane components supports the concept that the physiological shedding process continuously seeds cell surface moieties systemically. Occurrence in nor-

Table 2. Levels of four cell surface components estimated by the RDIH method in normal human plasma or sera

	Antibodies			
	Anti β2M	Anti FN	Anti HLA	Monoclonal antibody F_4
No. of samples tested	43	23	18	16
Dilution of plasma	1:24	1:100	1:16	1:2
Mean radius of heamolytic zone (μm)	1880 ± 630	2320 ± 340	1590 ± 180	880 ± 230
μg antigen/ml undiluted plasma	1.92 ± 0.70	311 ± 75	N.S.[a]	N.S.[a]

[a] No standard available

mal human plasma of the substances examined (except HSSA) has been known for some time (Mollison [19]; Allison et al. [1]; Evrin and Wibell [9]; Mosseson [21], but sparse attention has been given until now to the ubiquity of their presence or to their origin.

Ceriani and coworkers also recently reported the occurrence of normal cell surface antigens of human mammary epithelial cells in the circulation of athymic mice carrying breast cancer implants [28]. The antigens have also been observed in the circulation of normal women with benign breast disease, primary breast tumours, and other neoplasms, and in much elevated amounts in the sera of patients with metastatic breast malignancies [6].

It is probable that most, if not all, antigens associated with the neoplastic state (tumour associated antigens) are in fact normal cell constituents, expressed in heightened amounts or associated with certain stages of cell differentiation and maturation [31]. Elevated plasma levels of even normal cellular components could well be indicative of pathological conditions. Uncontrolled cell multiplication, as in neoplastic disease, can be expected to lead to the shedding of much greater than usual quantities of surface molecules. Thus, Ceriani et al. [6] found that women with metastatic breast disease exhibited high serum levels (110–280 ng/ml) of a normal epithelial cell surface antigen, whereas the concentration was low (ca. 15–30 ng/ml) in the sera of normal donors and of patients with localized breast neoplasia and other diseases. Quantification of normal cell substances in the circulation might come to have considerable diagnostic and prognostic significance (Sulitzeanu [29]).

The continuous presence of cell surface constituents in the circulation may have diverse physiological significance [2, 8, 26]. One function may be the prevention of autoimmune disease, the shed materials serving to block specific antibody and immunocyte reactions directed against normal self determinants. It is conceivable that tumour cell escape from immune attack rests, in part, on pre-emption of the normal process of shedding of normal cell compounds.

Table 3. Levels of blood group substance antigens estimated by the RDIH method in normal human plasma or sera

Samples from blood group donors (type)	No. of donors tested[a]	Antibodies	
		Anti blood group substance A	Anti blood group substance B
		Radius of haemolytic zone (μm)	
A	16	1530 ± 120	0
B	14	0	1560 ± 160
AB	6	1310 ± 50	1420 ± 60
	3	1280 ± 30	0
	4	0	1350 ± 40
0	9	0	0
	3	970 ± 60	1070 ± 60

[a] All sera or plasma diluted 1:5

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