Tumorigenic Potential and the Molecular Mechanism of the Carcinogenic Effect Exerted by 2-Nitroanisole

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Abstract. *The host-mediated in vitro/in vivo assay system was used to evaluate the tumorigenic potential of the aromatic nitro compound 2-nitroanisole (2-NA). After intraperitoneal administration of the compound, resident macrophages were recovered by peritoneal lavage from treated and untreated mice and cultured in soft agar. 2-NA was shown to be carcinogenic, and the tumorigenic potential was evaluated. Additionally, by establishment of a transformed peritoneal macrophage cell line, the underlying molecular mechanism of 2-NA's carcinogenic effect was studied.*

Aromatic nitro compounds have potent toxic and carcinogenic properties and, therefore, represent a considerable danger to humans (1, 2). They have a widespread distribution and can be found in chemical industry workplaces, as well as in emissions from diesel and gasoline engines, *etc*. Knowledge of the physiological significance of aromatic nitro compounds in humans is scarce (3). 2-Nitroanisole (2-methoxynitro-benzene, 2-NA), an important member of this compound family, is a mutagenic intermediate used as a precursor in the synthesis of 2-methoxyaniline (o-anisidine) in the manufacture of azo dyes (4). 2-NA exhibits carcinogenic activity in rodents, causing neoplastic transformation in the urinary bladder and, to a lesser extent, in spleen, liver and kidneys (4, 5). 2-NA exerts also a toxic effect, notably causing anaemia characterized by cyanosis due to increased levels of methemoglobin and accelerated destruction of erythrocytes (4). 2-NA has especially become of interest when an industrial accident in the Hoechst company in Germany in 1993 caused a large-scale leakage of 2-NA and subsequent local and regional contamination. 2-NA was the largest individual component (25%) of the fall-out mixture released by the accident. Various dermatological alterations were observed among children living in the area of the accident (6). Firefighters working at the site of the accident showed an induction of single- and double-strand breaks in mononuclear blood cell DNA (7). It has not been determined exactly whether 2-NA is a genotoxic or epigenetic carcinogen. In spite of its potent rodent carcinogenicity, 2-NA is weakly mutagenic in the Ames test with the *Salmonella typhimurium* TA100 strains (4). This carcinogen also exhibits a low activity in cytogenetic tests; at higher concentrations it induces a slight increase in chromosomal aberrations and sister chromatid exchanges (4).

The host-mediated *in vivo/in vitro* assay developed by our group enables an appraisal of the compound's carcinogenic potential (8,9). In the present study, the carcinogenic potential of 2-NA in the host-mediated *in vivo/in vitro* assay was investigated. Establishment of a 2-NA transformed permanent peritoneal macrophage cell line (Aona cells) from this host-mediated *in vivo/in vitro* assay allowed us to investigate the molecular mechanisms of the 2-NA mediated carcinogenic effect.

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Materials and Methods

Host-mediated in vivo/in vitro assay. All animals were 8-weeks-old male mice of the inbred NMRI strain weighing approximately 30 g. They were obtained from the Central Breeding Laboratories of the University of Frankfurt, Germany, and were maintained under specific pathogen-free conditions. They had free access to standard diet (Altromin) and water.

All chemicals used were reagent grade and were dissolved for each experiment immediately before use.

On day 0, 125 μg lipopolysaccharide (Sigma, Taufkirchen, Germany, LPS from E. coli serotype no. 0127:138), dissolved in 1 ml phosphate-buffered saline (PBS), was administered under aseptic conditions to each mouse intraperitoneally (*i.p.*). LPS are known to activate macrophages (10). 2-NA, was dissolved in 0.5 ml peanut oil containing 100 ng 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma). This cocktail was then administered on day 4 *i.p.* Control animals were treated using 100 ng TPA in PBS without 2-NA.

Macrophages were collected 4 days later by repeated peritoneal lavage. The approximate yield of macrophages per mouse was 2-4x10^6 cells. The suspended peritoneal macrophages were centrifuged at 1,000 xg for 10 minutes, resuspended and washed twice using 5 ml cell culture medium (up-medium) containing two-thirds DMEM (Sigma) with 10% fetal calf serum, 1% penicillin/streptomycin stock solution (containing 10^4 I.U./ml penicillin and 10^4 μg/ml streptomycin) and one third conditioned DMEM medium (CSF). The production of the conditioned DMEM medium has been described previously (9).

One half of the resuspended cells (2.5 ml per mouse) were plated into a sterile culture bottle (one bottle/mouse, 50 ml, 25 cm^2, Nunc, Wiesbaden, Germany), supplemented by the same volume of up-medium. The bottles were incubated at 37˚C in an atmosphere containing 5% CO_2. Non-adhering cells were removed 24 hours later by changing the medium. Metabolically acidified medium was replaced by the conditioned medium within the first week. Acidified medium was then replaced by DMEM medium containing 10% fetal calf serum and 1% of penicillin/streptomycin stock solution.

The second half of the suspension was transferred into soft agar as follows. One 24-well plate was used for each mouse. First, 0.2 ml of underlayer was pipetted into each well. After its solidification, 0.2 ml of upperlayer with the peritoneal macrophages at a density of 2-4x10^6 cells/ml was added. The plates were then incubated at 37˚C in a water-saturated atmosphere containing 5% CO_2. After 24 hours, 0.2 ml of conditioned medium was added per well and 5 days later the growth of cell colonies was evaluated. Under layer: 0.6% agar (Difco, Detroit, USA); 59.4% DMEM (1% penicillin/streptomycin); 20.0% fetal calf serum; 20.0% conditioned medium (containing CSF). Upper layer: 50.0% up-medium (containing peritoneal macrophages); 29.4% DMEM (1% penicillin/streptomycin); 0.6% agar; 20.0% fetal calf serum.

Cellular characterization of the transformed cell line (Aona cells): proof of Fc receptors. While FcR I and FcR II are expressed on the cellular surface of cells belonging to the leukocyte lineage, FcR III is expressed by macrophages solely. In order to detect the expression of the receptor on the cellular surface of the cell line established from our host-mediated *in vivo*/*in vitro* assay after transformation with 2-NA, normal macrophages and transformed cells were incubated in 24-well plates over night. Washing with PBS was followed by an incubation of one half of the cells with sheep red blood cells (SRBC) and the other half with opsonized SRBC. The number of bound erythrocytes was determined microscopically after thorough washing.

Cellular characterization of the transformed cell line (Aona cells). proof of Mac-1 antigen. Mac-1 is the only antigen expressed solely by macrophages. For the proof of Mac-1 expression of the surface of the cell line transformed by 2-NA, monoclonal antibodies of cell culture supernatants of M1/70 hybridoma cells were used. Mac-1 antigen reacts with the monoclonal antibody, resulting in a positive immunohistochemical response (Figure 2).

Mac-1 antigen has been described first in 1979, when Springer *et al.* reported that antibodies produced by M1/70 hybridoma cells bind to surface antigens, expressed only by cells of the monocyte lineage (11).

Immunocytochemical detection of the Mac-1 antigen was performed by the peroxidase-antiperoxidase reaction (PAP) according to Lansdorp *et al.* (12).

Non-specific esterase activity. Expression of non-specific esterases in 2-NA transformed macrophages was performed microscopically according to the method described by Bozdech and Baiton (13).

Proliferation characteristics of 2-NA transformed cells (Aona cells). Normal macrophages under serum-free conditions stop growing, while addition of serum leads to an alteration of gene expression with transition from G0 into G1 phase of the cell cycle. The ability of transformed cells to proliferate under serum-free conditions has been shown before, so here for the 2-NA transformed cells in comparison to normal macrophage growth behavior was examined under 10% FCS containing, serum-free and conditioned medium (colony-stimulating-factor) conditions.

For proliferation studies normal macrophages and 2-NA-transformed cells were seeded in 3 different 24-well plates at 2x10^4 cells/well. First, cells were cultivated in DMEM including 10% FCS for 1 week. Subsequently, in each plate, 8 wells were further cultivated with DMEM +10% FCS, 8 wells with serum-free medium and 8 wells with conditioned medium. After 3, 7, 14 and 21 days cell numbers were determined.

Oncogenic potential of 2-NA transformed cells (Aona cells). In order to study the oncogenic potential of the established cell line (2-NA transformed) 1x10^6 cells were suspended in PBS and injected subcutaneously at 2 different locations of the back skin of nude mice (nu/nu). Tumor growth was evaluated after 3 weeks. Five weeks after injection the animals were killed in order to perform histological analysis of the tumors.

Histological characterization of resulting tumour tissue. After extirpation of the tumors conservation occurred in formalin in order to perform histological examination. Following fixation in paraffin and haematoxylin-eosin staining, the tissue sections were analyzed under the light microscope (100-fold magnification).

Protein expression in the transformed cell line. Native PAGE, SDS-PAGE. Determination of protein content in complex mixtures was performed according to Bradford (14). 1-Dimensional SDS-PAGE was performed according to the method used by Schagger and von Jagow (15). Highly complex protein mixtures were separated using linear gradient (5-12%) separation gels (12x16 cm) and 4% stacking gels. Electrophoresis was performed constantly at 40 V within the stacking gel, at 80 V within the separation gel. Native PAGE used the same protocol for preparation of running buffer, sample buffer, separation and stacking gel without addition of SDS or b-mercaptoethanol. Gels were stained with Coomassie-Brillant Blue (16) or silver stain (17).

Activity of proto-oncogenes in the 2-NA transformed cell line (Aona cells). Oncoprotein activities in normal macrophages and 2-NA transformed cells were determined by means of an ELISA method. Here, the antigen (c-fos, c-myc, c-jun in the reduced cellular supernatants) was coupled on a plastic surface in a non-covalent manner. Free potential binding sites at the plastic surface were blocked using a 1% bovine serum albumin solution. Primary polyclonal antibodies against c-fos, c-myc and c-jun proteins were targeted at the antigens bound; the latter were then detected by means of a second enzyme-labeled antibody. Changes in extinction were measured by means of ELISA readers.

Results

Transforming potential of 2-NA. The evaluation system applied in the host-mediated *in vivo/in vitro* assay combines two important features: clone size and the respective clone frequency. As shown earlier (8), the carcinogens induce either large clones (more than 50 cells per clone), a large number of small clones (15-50 cells per clone) or both types of clones. An integrated evaluation of both clone size and clone frequency is done by multiplying the frequency of clones in each class by a factor based on the significance of clone size. This factor is calculated by division of the mean cell number of one clone by the mean cell number of the first significant clone. The class C3 with 15-19 cells per clone, which could be found in only 25% of the control animals, was designated as the first significant class. Classes C0-C2 (1-14 cells per clone) were designated non-significant, and therefore did not contribute to the transforming potential.

Microscopically distinguishable clone sizes were divided into ten classes (C0-C9, see Table I). The frequencies of clone sizes of a defined class were determined for each 24-well plate after an incubation period of 5 days. They were related to the cell number and were represented as indicated in Table I. The transforming potential of 2-NA was determined as follows. The microscopically determined frequency of the clone size of each class was multiplied with a factor taking account the significance of the clone size (Table I). The resulting products of classes C0-C9 were summed for each 24-well plate (results of one animal) separately. The median of each experimental group consisting of six animals and representing six 24-well plates designated the transforming potential of the respective substance.

In the host-mediated *in vivo/in vitro* assay with peritoneal macrophages, 2-NA was tested at three different concentrations corresponding to 0.1%, 5% and 10% of the compound's LD_{50}. The LD_{50} of 2-NA of 1,300 mg/kg was determined from the safety data sheet supplied by Sigma.

Twenty animals were divided in 4 groups, where 3 groups were treated with 2-NA and one group served as control. In all 2-NA treated groups clones could be detected, corresponding in size and number with the concentration of the applied compound. In repeated experiments it was shown that 2-NA at a concentration of 0.1% of its LD_{50} lead to a transformation of peritoneal macrophages in our assay. At a concentration of 0.1% of its LD_{50}, colony building activity could be observed in 3 plates. The calculated transforming potential was 1.0. At a concentration of 5% of its LD_{50} a transforming potential of 2.44 was calculated, and at 10% of the LD_{50} the transforming potential was calculated to be 3.75 (Table IIa). The transforming potential of 2-NA was determined using the median of the transforming potentials of single test animals.

Table I. *Definition of the classes for the clone size and their factors.*

Class	Cells per clone	Mean cell number per clone	Factor*
C0	0-4	2	0.0
C1	5-9	7	0.0
C2	10-14	12	0.0
C3	15-19	17	1.0
C4	20-24	22	1.3
C5	25-29	27	1.6
C6	30-49	40	2.4
C7	50-69	60	3.5
C8	70-99	85	5.0
C9	100+	125	7.4

*The factor is calculated by dividing the mean cell number of a clone from a class by the mean cell number of the first significant class. The class C3 with 15-19 cells per clone was designated as the first significant class. Classes C0-C2 were designated as not significant and does therefore not contribute to the transforming potential (factor 0).

At a concentration of 10% of the LD_{50} an immortal cell line could be established from the peritoneal macrophages. After approximately 4 weeks, a clonal expansion of the cells, cultured in 25 cm^2 culture flasks, parallel to their culture in soft agar, was observed (Figure 1a, b).

Cellular characterization of the obtained transformed cell line (Aona cells). In order to show a monocyte/macrophage origin of the immortalized cell line transformed by 2-NA, the Fc-receptor was examined. The Fc-receptor, a characteristic monocyte cell marker, enables monocytes to bind and opsonize immunoglobulin-loaded cells, and the macrophage-specific Mac-1antigen.

Fc-receptor (FcR). Evaluation of the number of opsonized and non-opsonized sheep red blood cells (SRBC) by the established cells transformed by 2-NA and normal macrophages respectively was performed microscopically. The numbers of bound erythrocytes per cell are shown in Table III. Specific binding of more than 5 erythrocytes was observed in 33.6% of the transformed cells, while non-specific binding of more than 5 erythrocytes was 3.3%. Corresponding binding values for normal macrophages were 59.5% (specific binding) and 27.2% non-specific binding.

Table IIa. *Peritoneal macrophage colony formation in soft agar after cellular transformation with of 2-nitroanisole at 3 different concentrations.*

No. of plate	Control / Test compound	Frequency of clone size									
		Non-specific		Specific							
		5-9	10-14	15-19	20-24	25-29	30-49	50-69	70-99	>100	Transforming potential of 2-nitroanisole
K1	Control:	-	-	-	-	-	-	-	-	-	
K2	0.5 ml	-	-	-	-	-	-	-	-	-	
K3	peanut	-	-	-	-	-	-	-	-	-	0.00
K4	oil +	-	1	-	-	-	-	-	-	-	
K5	100 ng TPA	-	1	-	-	-	-	-	-	-	
S1	2-nitroanisole	-	1	-	-	-	-	-	-	-	
S2	(0.1% of LD_{50}):	-	1	-	-	-	-	-	-	-	
S3	0.5 ml	-	-	1	-	-	-	-	-	-	1.00
S4	peanut oil +	-	1	-	-	-	-	1	-	-	
S5	0.052 mg 2-nitroanisole + 100 ng TPA	-	-	1	1	-	2	3	-	-	
S1	2-nitroanisole	-	-	-	-	-	-	-	1	-	
S2	(5% of LD_{50}):	-	-	-	-	-	-	-	-	3	
S3	0.5 ml	-	-	-	-	-	-	-	-	1	2.44
S4	peanut oil +	-	-	-	-	-	3	1	4	11	
S5	2.6 mg 2-nitroanisole + 100 ng TPA	-	-	-	-	-	-	-	1	8	
S1	2-nitroanisole	2	-	-	-	-	2	1	-	1	
S2	(10% of LD_{50}):	-	-	1	2	-	1	-	-	-	
S3	0.5 ml	2	-	2	-	-	1	-	1	-	3.75
S4	peanut oil +	-	-	-	3	-	-	-	1	-	
S5	5.2 mg 2-nitroanisole + 100 ng TPA	1	1	-	1	2	1	-	-	1	

Table IIb. *Peritoneal macrophage colony formation in soft agar after administration of 2-nitrophenole and 4-nitroanisole at a concentration of 10% of the LD_{50}.*

No. of plate	Control / Test compound	Frequency of clone size									
		Non-specific		Specific							
		5-9	10-14	15-19	20-24	25-29	30-49	50-69	70-99	>100	Transforming potential of 2-nitroanisole
K1	Control:	-	-	-	-	-	-	-	-	-	
K2	0.5 ml	-	-	-	-	-	-	-	-	-	
K3	peanut oil +	-	-	-	-	-	-	-	-	-	0.00
K4	100 ng	-	1	-	-	-	-	-	-	-	
K5	TPA	-	0	-	-	-	-	-	-	-	
S1	2-nitrophenole	-	-	-	-	-	-	-	-	-	
S2	(10% of LD_{50}):	-	-	-	-	2	4	1	3	1	
S3	0.5 ml peanut oil +	-	-	-	-	-	-	-	1	2	4.75
S4	5,2 mg	-	-	-	-	-	-		13	1	
S5	2-nitrophenole + 100 ng TPA	-	-	-	2	-	2	-	-	1	
S1	4-nitroanisole	6	-	-	-	1	2	1	-	-	
S2	(10% of LD_{50}):	-	-	2	-	-	1	-	-	-	
S3	0.5 ml peanut oil +	1	-	-	-	-	-	-	-	-	0.00
S4	2.8 mg	2	-	-	-	-	-	-	-	-	
S5	4-nitroanisole + 100 ng TPA	-	-	-	-	-	-	-	-	-	

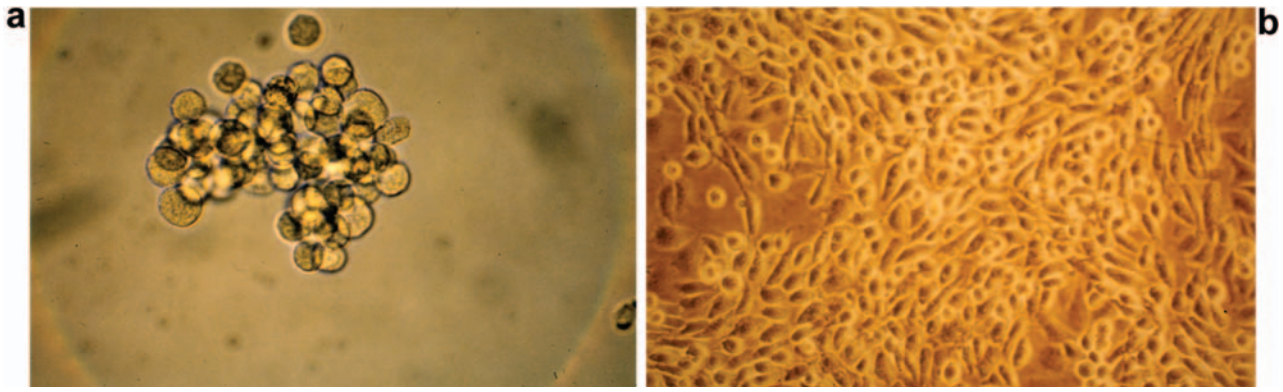

Figure 1. *Photomicrograph of peritoneal macrophage clonal expansion following transformation with 2-NA; (a) clone of approximately 50 cells in soft agar (700 fold magnification); (b) macrophages after 2 weeks of cultivation in flasks (700 fold magnification).*

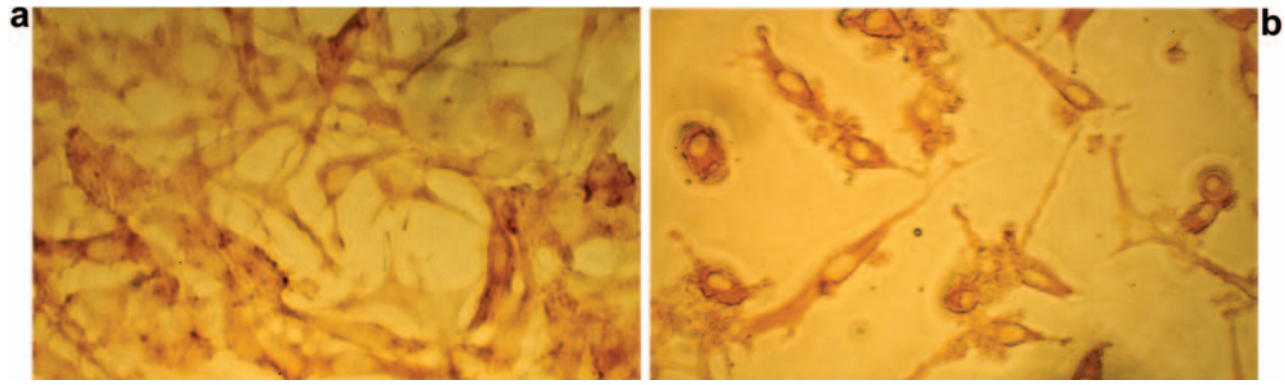

Figure 2. *Photomicrograph of 2-NA transformed peritoneal macrophages (350 fold magnification) (a) and normal macrophages (1000 fold magnification) (b) following the Mac-1 assay.*

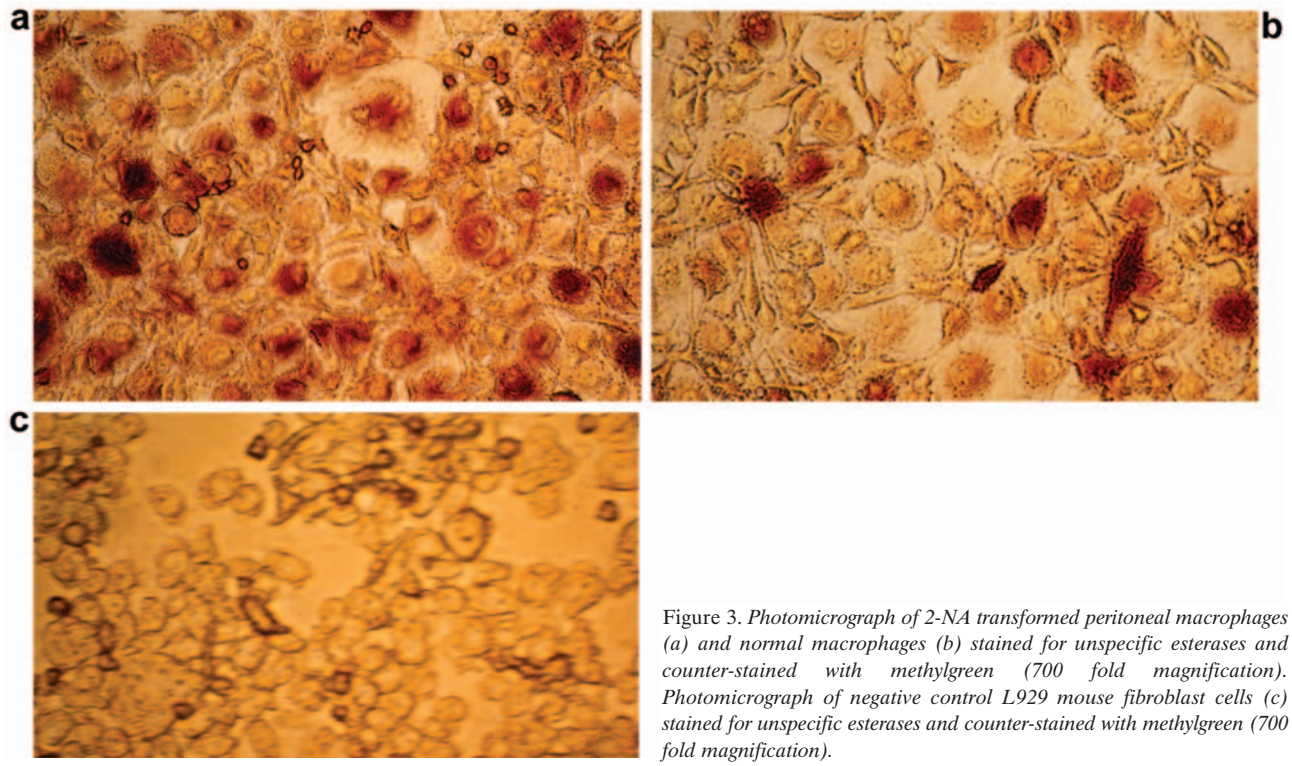

Figure 3. *Photomicrograph of 2-NA transformed peritoneal macrophages (a) and normal macrophages (b) stained for unspecific esterases and counter-stained with methylgreen (700 fold magnification). Photomicrograph of negative control L929 mouse fibroblast cells (c) stained for unspecific esterases and counter-stained with methylgreen (700 fold magnification).*

Table III. *Binding of opsonized and non-opsonized sheep red blood cells (SRBC) by 2-NA transformed cells and normal macrophages.*

Cells	SRBC	Percentage of bound erythrocytes per cell [%]			
		0	1-4	5-9	>10
2-NA transformed	opsonized	18.8	47.8	24.8	8.8
2-NA transformed	non-opsonized	35.4	61.3	3.3	0
normal macrophages	opsonized	7.6	32.9	23.5	36
normal macrophages	non-opsonized	24.3	48.5	21.3	5.9

These results demonstrated that a part of the transformed cells did not express Fc receptors and that Fc-receptor mediated phagocytosis was diminished compared to normal macrophages.

Mac-1 antigen. Normal macrophages as well as 2-NA transformed cells showed a 100% expression of Mac-1 antigen (Figure 2).

Non-specific esterase activity. Non-specific esterases can be additionally used for proof of the monocytic origin of a cell. This is due to the fact that monocytic cells express non-specific esterases with fluoride sensitivity in contrast to esterases of other leukocytes (13).

The percentage of cells with non-specific esterase activity in normal macrophages was 100%, but 2-NA transformed cells were 35% positive for non-specific esterase and the negative control mouse fibroblast cell line L-929 showed no activity (Figure 3 a, b, c).

Morphology of 2-NA transformed cells (Aona cells). The transformation process is accompanied by characteristic morphologic alterations of cells, induced by alterations in the expression of structural proteins and certain functional proteins, especially those involved in growth regulation. Properties especially prown, are alterations of cell culture density, decrease or complete loss of adherence and growth independent of serum or growth factors. An additional characteristic is the ability of those cells to induce tumors in nude mice.

The most striking morphological characteristics 2-NA transformed cells are larger size than normal macrophages and a diminished adherence in culture.

Proliferation characteristics of 2-NA transformed cells (Aona cells). Transformed cells lose the ability to regulate growth during the transformation process. They also grow at conditions, where normal cells stop growing or die, such as lack of growth factors or serum and contact inhibition.

In contrast to normal macrophages, 2-NA transformed cells show continuous proliferation with serum-free medium, medium supplemented with 10% FCS and with conditioned medium (Figure 4 a, b).

Oncogenic potential of 2-NA transformed cells (Aona cells). Different transformed murine cells are able to induce tumors in nude mice (nu/nu mice) independent of their origin (18, 19). Transformation of murine peritoneal macrophages by 2-NA resulted in visible tumor formation 3 weeks after injection at 2 different locations on the back of the test animal. Animals were killed 5 weeks after injection for histological characterization of the tumors. Macroscopically the tumors were clearly shaped and showed no signs of local or generalized metastasis. The blood supply to the tumors was excellent (Figure 6).

Histological characterization of resulting tumour tissue. After extirpation of the tumors the tissues were analyzed under the light microscope (Figure 7). Typical signs of malignant tumors were observed. Atypical chromatin-rich cell nuclei with increased frequencies of mitosis and an alteration of the nucleus-plasma relation were detected. The microscopic appearance suggested that the tumors were of mesenchymal origin: histologically small, anaplastic malignant cells with multiple mitoses, spindle-cell shaped cells and chromatin-rich nuclei with enlarged nucleoli. This corresponds to the finding that 2-NA transformed peritoneal macrophages induce sarcoma in immuncompetent nude mice.

Biochemical characterization of the transformed cell line (Aona cells)

Protein expression. Native PAGE, SDS-PAGE. In contrast to oncoviruses, chemical and physical carcinogens are not able to introduce new genetic material into the affected cell. Therefore, all characteristic alterations in protein expression following chemical carcinogenesis are related to interference with the activity of already present genes. Protooncogenes under physiological conditions are either silent or expressed at a very low level. Activation of cellular protooncogenes within the scope of chemical carcinogenesis can lead to overexpression of specific oncoproteins with growth-and transformation-regulating qualities. Alteration of growth behavior, adherence properties and the fact that macrophages *per se* belong to a group of cells with high secretory potential, lead us to protein biochemical analysis of the serum-free supernatants of these cells. Following sterile filtration, reduction and dialysis, the supernatant material was analysed electrophoretically.

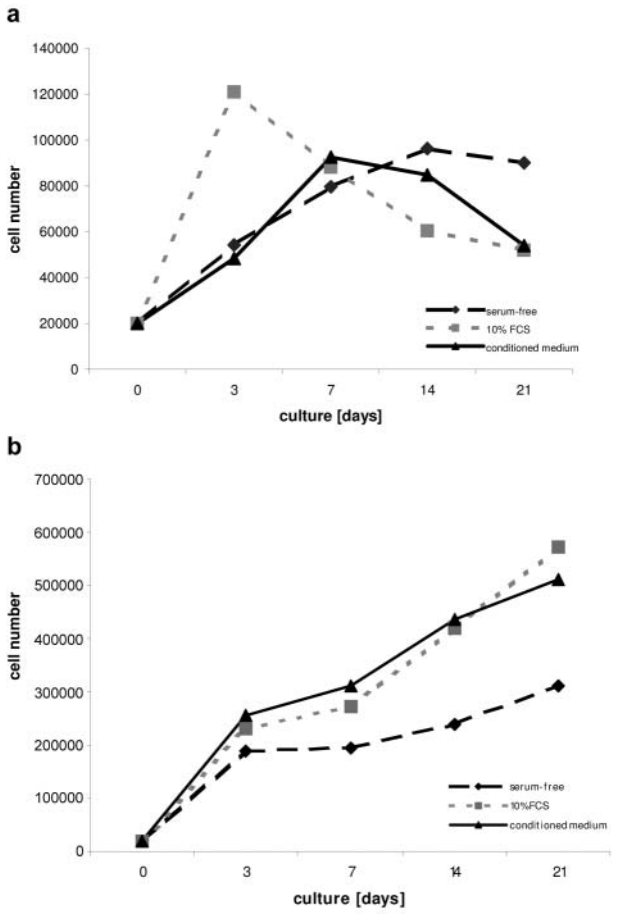

Figure 4. *Proliferation of normal macrophages (a) and 2-NA transformed cells (b) under serum-free conditions, under 10% FCS conditions and with conditioned medium.*

Under non-denaturating conditions one-dimensional analysis of 2-NA transformed cell supernatant revealed significant expression of additional proteins not found in normal macrophages. The first prominent new protein showed a molecular weight of 66 kDa, the second protein ranged between 66 and 132 kDa (Figure 5a). In order to obtain additional information on these 2 proteins, electrophoresis was also performed under denaturating conditions. Compared to normal macrophages, especially in the low molecular weight range between 14.4 and 45 kDa, expression of additional proteins was observed (one prominent band at 45 kDa, four bands between 31 and 45 kDa, one band between 21.5 and 31 kDa). In the high molecular weight range, no additional protein expression could be detected (Figure 5b). In conclusion, similar to the findings of Schlatterer *et al.* (20, 21) expression of transformation-specific proteins by 2-NA transformed macrophages was observed.

Activity of proto-oncogenes in the 2-NA transformed cell line (Aona cells). In the context of disturbances of cellular growth and proliferation during chemical carcinogenesis, the 3 transcription factor groups; c-jun, c-myc and c-fos play an important role. Dysfunction of these proto-oncogenes has been shown in diverse human tumors like lymphoma or sarcoma (22, 23). For this reason, the activities of cellular nuclear oncoproteins c-fos, c-jun and c-myc have been investigated in this study for further characterization of the macrophage cell line obtained following transformation by 2-NA. Reduced cellular supernatants were examined for oncoprotein activity. The expression of individual oncoproteins in normal macrophages was defined as reference and set at 100%, and the oncoprotein expression levels in 2-NA transformed macrophages were compared against normal expression. In ELISA assays c-fos and c-myc showed significant down-regulation in the transformed cells as compared to normal macrophages, and c-jun was clearly overexpressed (Table IV). This overexpression could be shown to be concentration-dependent, *i.e.* dependent on the amount of supernatant protein applied in the ELISA test in contrast to c-fos and c-myc.

Structure-activity relationship between 2-NA and mono-nitrobenzene. To study the structure-activity-relationship with regard to carcinogenic activity, 4-nitroanisol and 4-nitrophenol were also examined in the host mediated *in vivo/in vitro* assay (Table II b). In both cases, the substances were tested under the same conditions and at a concentration of 10% of the LD_{50}. In the case of 4-nitroanisol no transforming potential could be detected. 2-nitrophenol showed a transforming potential of 4.75 (Table II b), higher than that of 2-nitroanisol (Table II a). The LD_{50} of 2-nitrophenol is 1,297 mg/kg in mouse (Merck Index, 11.Edition 1989).

Discussion

The carcinogenic properties of 2-NA have been tested in a number of test systems. The host-mediated *in vivo/in vitro* assay, in addition to evaluation of a compound's carcinogenic potential, offers the opportunity to investigate underlying molecular mechanisms after successful establishment of transformed cell lines. It was shown that 2-NA demonstrates carcinogenic potential. Transformed Aona cells, established from the assay, induced tumors in nude mice and, therefore, possess oncogenic properties. These tumors show cellular and biochemical characteristics of malignant tumors, including atypical chromatin-rich nuclei, increased frequency of mitoses, and a shift in the nucleus-plasma ratio. Histologically the tumors resemble tumors of mesenchymal origin and they are able to induce sarcoma in nude mice.

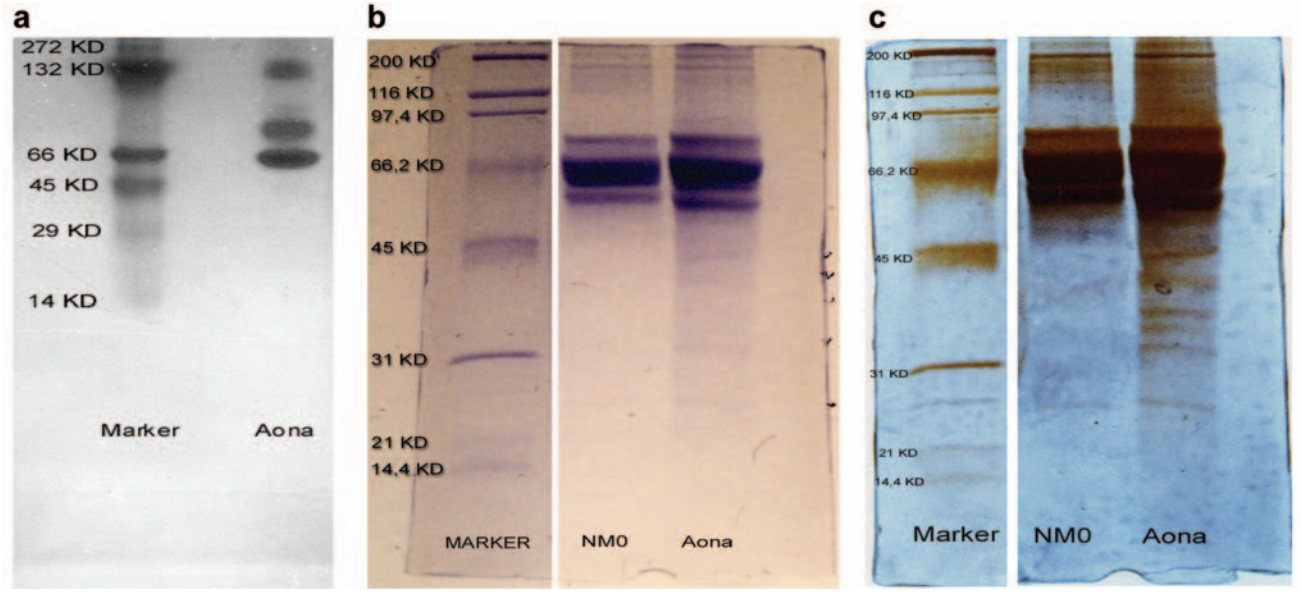

Figure 5. *1D-PAGE of the secretion profile of normal macrophages compared to 2-NA transformed macrophages; (a) native PAGE (Coomassie Brillant Blue), (b) SDS-PAGE (Coomassie Brillant Blue) 1D-PAGE of the secretion profile of normal macrophages compared to 2-NA transformed macrophages; (c) SDS-PAGE (silver stain).*

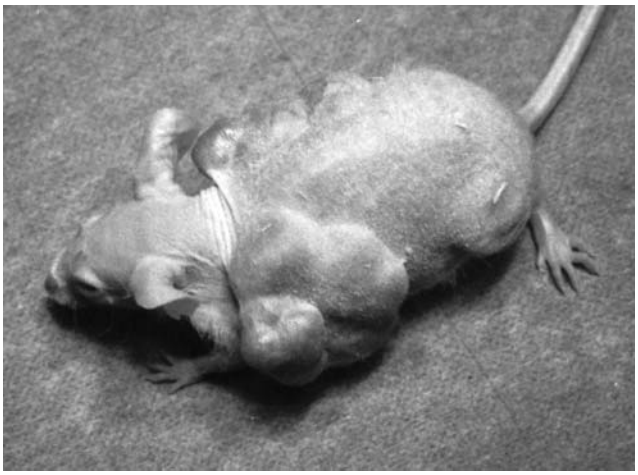

Figure 6. *Induction of tumors in nude mouse injected subcutaneously at two different sites each with 1x10^6 Aona cells. The figure shows the tumor growth after 6 weeks.*

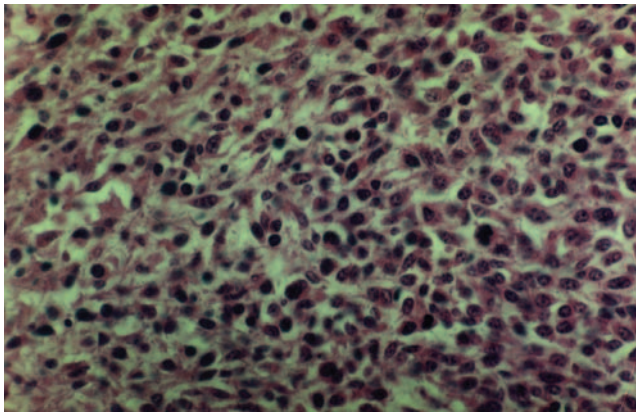

Figure 7. *HE-staining (1000 fold magnification) of a tumor induced by Aona cells in a nude mouse.*

Transformation of the cell line was shown. Typical morphologic alterations such as enhanced size and loss of adherence properties were observed. The latter property is generally associated with loss of production of extracellular matrix compounds. During the transformation process a separation of cell growth and differentiation is observed. Therefore transformed cells can be regarded as dedifferentiated. The latter is supposed to be the reason for reduced adhesion properties. In the transformed cell line investigated here, uncontrolled growth as compared to normal macrophages was found, also supporting all other observed properties of transformation. The cells transformed by 2-NA grew under conditions where normal cells do not grow, and, additionally, a 3-dimensional growth behaviour was observed in contrast to normal macrophages. This suggests a loss of growth regulation due to activation of proto-oncogenes. In the 2-NA transformed Aona cell line, an

Table IV. *Relative expression of c-jun, c-fos and c-myc in 2-NA transformed murine macrophages as compared to normal macrophages (100%) at different amounts of protein in the reduced cellular supernatants.*

	c-jun		c-fos		c-myc	
Protein content per well [ng]	Normal macrophages	2-NA transformed cells	Normal macrophages	2-NA transformed cells	Normal macrophages	2-NA transformed cells
400	100%	144%	100%	58%	100%	79%
800	100%	154%	100%	63%	100%	66%
1600	100%	166%	100%	59%	100%	80%
3200	100%	219%	100%	60%	100%	72%

Figure 8. *Structural formulae of 2-Nitroanisol, 2-Nitrophenol and 4-Nitroanisol.*

overexpression of the c-jun oncoprotein was shown. C-jun belongs to the group of "immediate early genes", therefore its regulation requires no protein synthesis and is performed by mitogenic stimulation at the transcriptional and post-transcriptional level (24, 25). This oncogene family is able to code for transcription-regulating proteins (26). The product of the c-jun proto-oncogene is able to form homodimers, showing high affinity to DNA with the basic amino acids of the c-jun protein responsible for strong binding to DNA (27). By this mechanism c-jun protein can transmit mitogenic signals directly to DNA. In addition to the overexpression of proto-oncogene c-jun, an up-regulation of low-molecular weight transformation-specific proteins was found in Aona cells. The existence of transformation-specific proteins in cellular supernatants, together with the proliferation under serum-free conditions suggests that these transformed cells are capable of producing certain factors essential for autonomous growth. This was confirmed by the fact that the proliferation rates of Aona cells in conditioned medium (CSF, colony stimulating factor) are higher than under normal conditions.

To study the structure-activity relationship, the carcinogenic activity of 4-nitroanisol and 2-nitrophenol with structural similarities to 2-NA was examined. Under similar conditions and at a concentration of 10% of the LD_{50} the transforming potential was 0.0 for 4-nitroanisol and 4.75 for 2-nitrophenol. Experiments with rats have shown that 2-nitrophenol is the main metabolite of 2-nitroanisol (28). For this reason, we postulate that the transforming potential of 2-nitroanisol, at least partly, is attributed to the carcinogenic activity of its main metabolite 2-nitrophenol.

On the other hand, it is evident that the carcinogenic activity is highly dependent on the molecular structure. In the case of 4-nitroanisol no transforming potential was observed, whereas 2-nitrophenol is a potent carcinogen. Since the substituents in 4-nitroanisol are in "para" position and the substituents in 2-nitroanisol and 2-nitrophenol are in "ortho" position, we hypothesize that the "para" position may not be suited to develop carcinogenic activity (Figure 8).

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