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SINGLE CELL ANALYSIS OF DNA MODIFICATIONS INDUCED BY CHEMICAL CARCINOGENS AND CYTOSTATIC DRUGS

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Summary. - *Antibodies recognizing specific DNA modifications allow the immunocytochemical visualization and quantification of these modifications at the level of the individual cell. Thus, the formation and repair of DNA adducts induced by chemical mutagens and carcinogens and by cytostatic drugs can be studied in very small samples in relation to e.g. cell type and tissue localization. A number of existing and potential applications in the fields of chemical carcinogenesis, chemical mutagenesis, experimental chemotherapy and molecular epidemiology are illustrated.*

Riassunto (Analisi a livello di singola cellula delle modificazioni del DNA indotte da cancerogeni chimici e farmaci citostatici). - *L'uso di anticorpi che riconoscono specifiche modificazioni del DNA permette la visualizzazione e la quantizzazione immunocitochimica di queste modificazioni a livello di cellule individuali. In questo modo è possibile studiare, in campioni molto piccoli, la formazione e il riparo di addotti al DNA indotti da agenti chimici mutageni e cancerogeni e da farmaci citostatici in relazione al tipo cellulare e alla localizzazione nei tessuti. Nell'articolo sono anche illustrate le applicazioni esistenti e potenziali di questa tecnica nel campo della cancerogenesi chimica, mutagenesi chimica, chemioterapia sperimentale ed epidemiologia molecolare.*

Introduction

Modification of cellular DNA has been linked with diverging biological effects, such as cell death, cytostatic effects, mutagenesis and carcinogenesis. Important parameters are the pattern and extent of DNA modification, the proliferative activity of the exposed cell, and its capacity to repair relevant DNA damage. The present paper reviews a specific, sensitive, and quantitative immunocytochemical method for the analysis of DNA damage at the single cell level. A number of existing and potential applications in the field of chemical carcinogenesis, chemical mutagenesis, experimental chemotherapy and molecular epidemiology will be discussed.

Immunocytochemical visualization of DNA-adducts

Several laboratories have developed immunocytochemical methods for the demonstration of specific DNA structures or DNA modifications induced by carcinogens and other agents in cultured cells [1-7], in animal tissue samples [7-17], or in human tissue samples [18]. Our division has concentrated on a double peroxidase-antiperoxidase staining assay, whereas others used fluorochrome-labeled (second) antibodies or streptavidin for the ultimate signal. One obvious advantage of an assay at the single cell level in comparison with the analysis of isolated bulk DNA stems from the heterogeneity in adduct formation and repair within tissues. This is important, since toxic and carcinogenic effects are often confined to particular cell types and specific sites within a tissue. Another advantage of an immunocytochemical approach is the small number of cells needed, down to a few hundred. This allows the identification of small subpopulations within a tissue, experiments on less extended tissues, and last but not least, the use of less invasive methods for the collection of human tissue samples. The present paper illustrates the above mentioned points and discusses a number of potential applications of the immunocytochemical detection of DNA modifications.

The peroxidase-antiperoxidase technique

The general outline of the staining procedure is as follows: freeze-substituted or cryostat sections are treated with methanol-H_2O_2 (to inactivate endogenous peroxi-

dase), RNAses A and T_1 (to remove carcinogen-RNA adducts), and alcoholic NaOH (to denature the DNA and make it more accessible to the immune reagents). After incubation with the first (rabbit) antiserum, directed against a particular DNA adduct, the presence of bound antibodies is visualized by PAP staining involving incubation with goat-antirabbit IgG and a peroxidase-(rabbit)antiperoxidase (PAP) complex. To increase sensitivity, the latter two steps are repeated *i.e.* double PAP staining. After a number of rinsing steps the peroxidase substrate 3,3-diaminobenzidine (DAB) is added together with imidazole and H_2O_2. The resulting product of the enzymic conversion of the diaminobenzidine is a stable, yellow-brown precipitate. A detailed protocol of the entire staining procedure for tissue sections has been published [14]. We have shown that this staining procedure can be used to visualize DNA modifications in a large variety of normal tissues in rats, mice and hamsters as well as in solid, transplantable tumors. The staining procedure has been adapted to cultured cells and other single cell preparations [7, 18].

Compared to other cytological methods, such as autoradiography after application of radioactively labeled DNA-modifying agents [19, 20], immunocytochemistry not only offers a superior sensitivity and specificity in visualizing well-defined DNA modifications (as opposed to the widespread reaction with protein detected by autoradiography), but also a better cytological resolution. Several immunocytochemical assays exist: the (double) PAP, immunofluorescence, and immunogold. At this moment, it is not clear which assay will be the best one with regard to sensitivity, specificity and simplicity. An advantage of the PAP assay, however, is the high stability of the reaction product, allowing repeated scanning and unlimited storage of stained samples.

Adducts which have been visualized; detection limits

The DNA adducts visualized immunocytochemically in our laboratory are listed in Table 1. All results reported in this paper have been obtained with conventional rabbit antisera. In some cases monoclonal antibodies from other laboratories have been tested and compared with the available antisera with regard to sensitivity and background staining. To date the rabbit antisera have proved at least as useful as the monoclonals. It has, however, been observed that monoclonal antibodies are able to discriminate between closely related DNA adducts or between different epitopes on an individual (bulky) DNA adduct ([21]; F.F. Kadlubar, personal communication). In particular, studies on modified DNA in which the modifying agent has not been fully identified, might benefit from the use of more specific antibodies.

Hetereogeneity in adduct formation

As an example of site-dependent heterogeneity in adduct formation within a single cell type we will review some data on rat liver. After treatment of rats with a single dose of dimethylnitrosamine (DMN) or diethylnitrosamine (DEN), a striking heterogeneity of the distribution of alkylated DNA bases (O^6-MeGua, O^6-EtGua, 7-MeGua) has been observed in this organ. Centrilobular hepatocytes were found to contain much more of these alkylated bases in their DNA than did hepatocytes in the periphery of the liver lobule (the portobiliary region). Fig. 1 illustrates the distribution of O^6-EtGua in the liver of a DEN-treated rat [11]; similar results were obtained for 7-MeGua and O^6-MeGua after DMN [22]. In other experiments with DMN and DEN, similar gradients between centrilobular and peripheral hepatocytes have been reported for other parameters like nitrosamine-induced cell death [23], distribution of radioactive nitrosamine (by autoradiography; [24]), and nitrosamine-induced unscheduled DNA synthesis [25]. Our results show that these gradients are due to a gradient in metabolic activation and not a consequence of differences in intrinsic sensitivity of centrilobular *v.* peripheral hepatocytes. This conclusion is strengthened by our observation [11] that the injection of rats with the direct ethylating agent ethylnitrosourea (ENU) gives rise to a homogeneous distribution of DNA alkylation in the

Table 1. - *DNA adducts visualized by double PAP staining*

Adduct	Abbreviation	Detection limit (adducts/10^7 nucleotides)
O^6-methylguanine	O^6-MeGua	10
O^6-ethylguanine	O^6-EtGua	10
7-methylguanine *(a)*	7-MeGua	100
guanine-8-(acetyl)aminofluorene *(b)*	Gua-8-(A)AF	4
guanine-N^2-benzopyrenediolepoxide *(b)*	Gua-N^2-BPDE	5
guanine-N7-aflatoxin B_1	Gua-N7-AFl	2
platin-DNA adducts		
cis-diamminedichloroplatinum(II)	cDDP-DNA	20
carboplatin (CBDCA)	CBDCA-DNA	nd *(c)*

(a) Antiserum from P. Degan, R. Montesano and C.P. Wild (*Cancer Res.* 1988. 48: 5065-5070).
(b) Antisera provided by E. Kriek and F.-J. Van Schooten.
(c) Not determined.

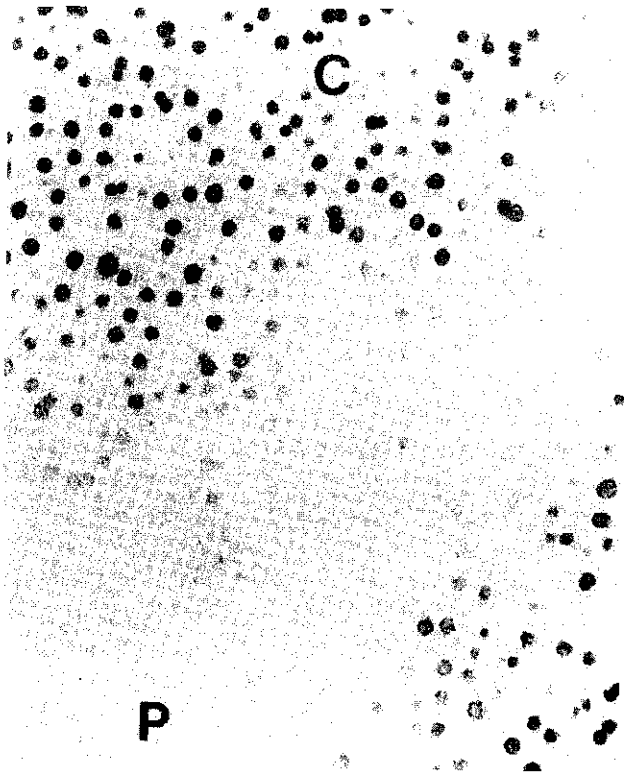

Fig. 1. - Localization of O6-EtGua in DNA in liver nuclei of a DEN-treated rat (5 h after 50 mg/kg). C: central vein; P: portal tract (from Menkveld *et al.* [11], reproduced with kind permission).

liver (Fig. 2). Further experiments with rat liver showed that different cell types in this organ can also differ substantially with respect to their capacity to repair specific DNA alkylation products.

Hetereogeneity in DNA repair.

The repair of O6-EtGua has been investigated in tissues of the ENU-treated rat. ENU was selected since it is a directly acting ethylating agent, which induces rather similar levels of DNA ethylation throughout the animal [26]. At 2 and 6 h after injection, a relatively homogeneous distribution of O6-EtGua was observed in all tissues studied, including liver (Fig. 2) and pancreas. When, however, sections of the latter two tissues were stained for O6-EtGua 24 h after injection, striking heterogeneities were observed. In the liver, levels of O6-EtGua in hepatocytes had fallen below the detection limit, whereas non-parenchymal cell types still contained significant levels of this modified base (Fig. 3). The heterogeneity in O6-EtGua-specific staining at 24 h after injection was even more pronounced in the pancreas (J. Bax, unpublished result). Nuclei in the islands of Langerhans had retained most of their O6-EtGua during the 24 h period after injection, whereas a sharp decline was observed in the O6-EtGua levels in the acinar cells (Fig. 4). Our results on the liver are in agreement with those of Lewis and Swenberg [27, 28] obtained on DNA isolated from separated cell fractions. We conclude that the value of data on the formation and loss of DNA adducts derived from isolated total tissue DNA is limited if not combined with immunocytochemical analysis of the distribution of adducts within the tissue.

Studies on small populations of cells

Immunocytochemical analysis is of particular value when the (supposed) target tissue is small in size and/or when the cell type of interest is scattered throughout an entire tissue or organism. Examples are the different epithelia of the respiratory system and the gastrointestinal tract (some of which are highly specific targets for the carcinogenic action of a number of aldehydes and nitroso compounds), and the peripheral nerve system (unwanted target for a number of DNA damaging anticancer drugs). The advantages of the immunocytochemical analysis are illustrated by studies with the tobacco-specific nitrosamine NNK and the polycyclic hydrocarbon benzo(a)pyrene.

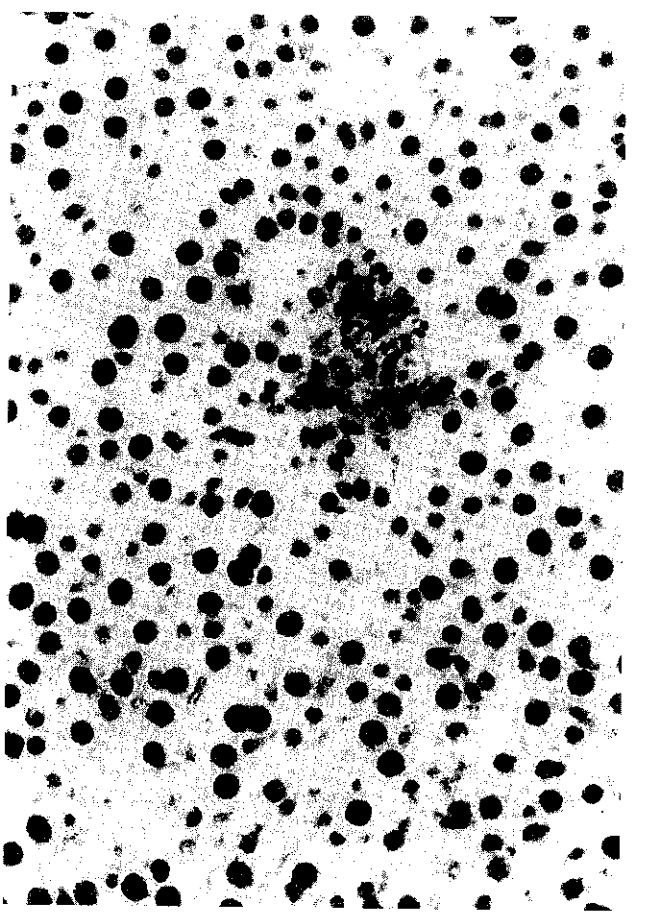

Fig. 2. - Localization of O6-EtGua in DNA in liver nuclei of an ENU-treated rat (2 h after 140 mg/kg). Note the homogeneous staining pattern including the area around the central vein (from Menkveld *et al.* [11], reproduced with kind permission).

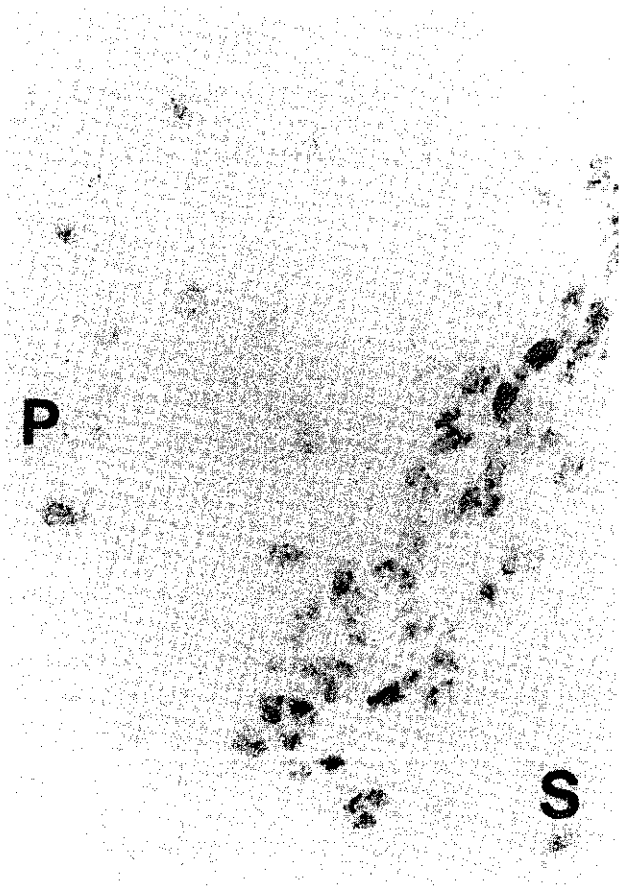

Fig. 3. - Localization of O6-EtGua in DNA in liver nuclei of an ENU-treated rat (24 h after 140 mg/kg). Staining of parenchymal cells has almost completely vanished, whereas sinusoidal cells (S) and vascular endothelium still show substantial staining (from Menkveld *et al.* [11], reproduced with kind permission).

Methylation of DNA in rat tissues by the tobacco-specific, carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). - Nitrosation of the tobacco alkaloid nicotine gives rise to the formation of NNK and some other tobacco-specific nitrosamines. These nitrosamines, found not only in tobacco smoke but also in smokeless tobacco, might play an important role in the etiology of tobacco-related human cancer [29]. Knowledge of the extent of DNA alkylation in potential target cells in the human respiratory tract, the bladder, renal pelvis, pancreas, cervix, and the upper part of the digestive tract, might yield important information on the contribution of these nitrosamines to the formation of tumors in humans. We studied NNK-induced DNA methylation in various tissues of the rat, in particular the respiratory tract. A special technique, embedding in methyl metacrylate, was introduced to allow the immunocytochemical visualization of DNA adducts in the nose, where bony structures are lined with thin layers of epithelium. Advantages of plastic embedding are the possibility to cut thinner (*e.g.* 2 μm) sections and the improved morphology, in particular of the nasal turbinates and other bony structures. A particular advantage of methyl metacrylate is that it can be removed after sectioning, making the DNA accessible to the adduct-specific antibodies. We observed a highly specific pattern of DNA methylation after a single intraperitoneal dose of NNK (5-40 mg/kg; Figs 5 and 6). O6-MeGua and 7-MeGua could be visualized in the nasal cavity, trachea, lung and liver. Heavily stained nuclei were observed in particular in the Bowman glands in the *lamina propria* of the olfactory region. Adduct-specific precipitate was also seen in other cells of the nasal epithelium: the goblet cells of the respiratory epithelium and - only after relatively high doses of NNK - the supporting and basal cells of the olfactory epithelium. Nuclear staining was also observed in the liver and the lung; in the latter organ the staining was restricted to the bronchiolar epithelium (Clara cells). It is of interest that NNK, via a different metabolic route, gives rise to 4-(3-pyridyl)-4-oxobutylation of cellular macromolecules. It is not known to what extent these two pathways contribute to tumor formation. Antibodies against 4-(3-pyridyl)-4-oxobutylated DNA might be useful to clarify

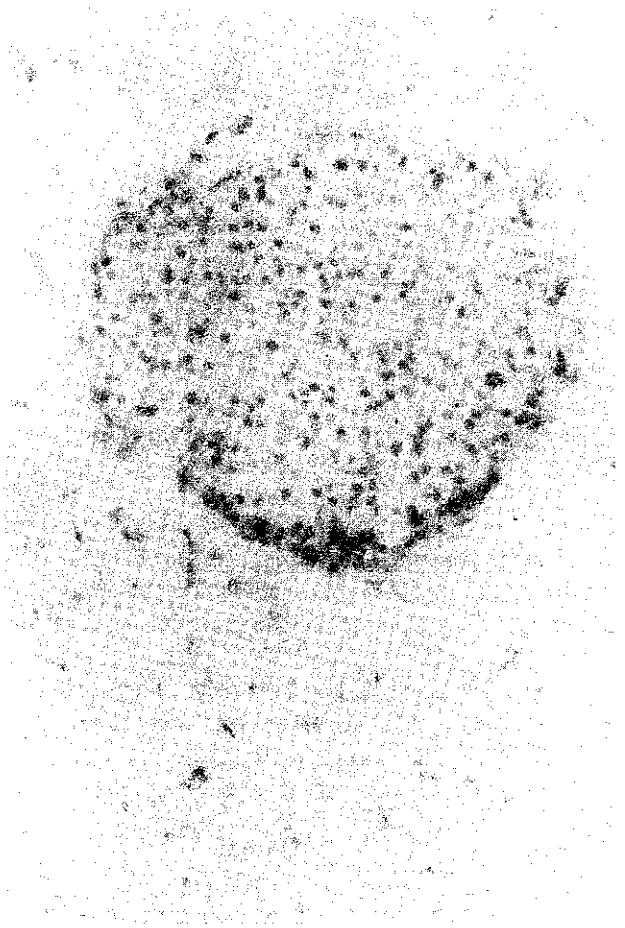

Fig. 4. - Localization of O6-EtGua in DNA in the pancreas of an ENU-treated rat, 24 h after injection. The nuclei in the islands of Langerhans still contain substantial levels of O6-EtGua, whereas the levels of this base have decreased sharply in the surrounding acinar cells.

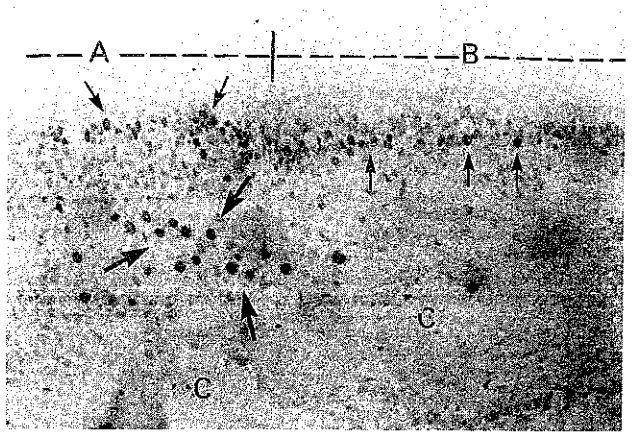

Fig. 5. - Localization of O^6-MeGua in DNA the olfactory (A) and respiratory (B) region of the ethmoid turbinate of the rat, 6 hour after NNK (30 mg/kg). In the olfactory epithelium the apical cells are stained (region A, small arrows), whereas in the respiratory epithelium the basal cells (region B, small arrows) are stained. Staining in the *lamina propria* is restricted to the Bowman glands of the olfactory region (large arrows). C: cartilage. Counterstaining with haematoxylin.

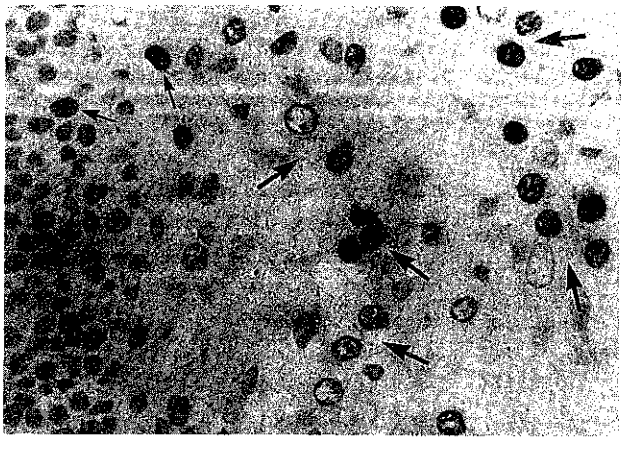

Fig. 6. - 7-MeGua-specific staining in the olfactory region of the ethmoid turbinate of the rat 6 h after NNK (30 mg/kg). The adduct is restricted to the glandular cells (large arrows) and the ductal cells (small arrows) of the Bowman glands. Counterstaining with haematoxylin.

the capacities of different cell types to perform both types of metabolic activation. The next step will be to investigate respiratory tissue of heavy smokers by means of immunocytochemistry for the presence of alkylated DNA.

Adduct visualization in human cells

cDDP-DNA adducts in buccal cells of cancer patients. - We have recently extended our rodent studies on the immunocytochemical visualization of cDDP-DNA and carboplatin-DNA adducts [7, 17] to cells obtained from cancer patients treated with cDDP or cis-diammine (1,1-cyclobutanedicarboxylato)platinum(II) (carboplatin), usually in combination with other drugs. The double PAP-technique, which had to be adapted to single cell-samples, proved to be sufficiently sensitive to visualize adducts in buccal epithelial cells of patients treated with doses down to 20 mg/m^2 cDDP or 350 mg/m^2 carboplatin. cDDP-DNA adducts could also be visualized in exfoliated bladder cells collected from urine, but further analysis seemed less attractive because of the relatively poor morphology of these cells. A third cell population, of which the isolated DNA has been studied by several authors [30, 31], is the peripheral leukocyte. It has been suggested that intrastrand cDDP-DNA adduct levels in peripheral leukocytes have predictive value with regard to tumor response [30]. Preliminary *in vitro* experiments in our laboratory (incubation for 1 h at 37 °C in medium containing 0 to 100 mg/l cDDP) showed that cDDP-DNA adducts are formed to a five times lower extent in human lymphocytes than in human buccal cells. This might make the lymphocyte a less attractive cell type for the monitoring of cDDP-induced adducts in humans. Results, obtained on a series of 14 patients, showed a significantly higher and dose-related nuclear staining intensity in buccal cells after treatment with cDDP than before treatment. It proved to be possible to monitor the cDDP-DNA adduct level in buccal cells from a single patient during a number of treatment cycles (Fig. 7). Since it is difficult to envisage how the formation and loss of DNA adducts in buccal cells or lymphocytes, might yield information on these two parameters in tumor cells, the next step will be the visualization and quantification of cDDP- and carboplatin-DNA adducts in human tumor biopsies. Hopefully, this will allow the development of an assay to predict the clinical result early during therapy. Preliminary experiments have already shown the feasibility of adduct visualization and quantification in rodent tumor samples [7].

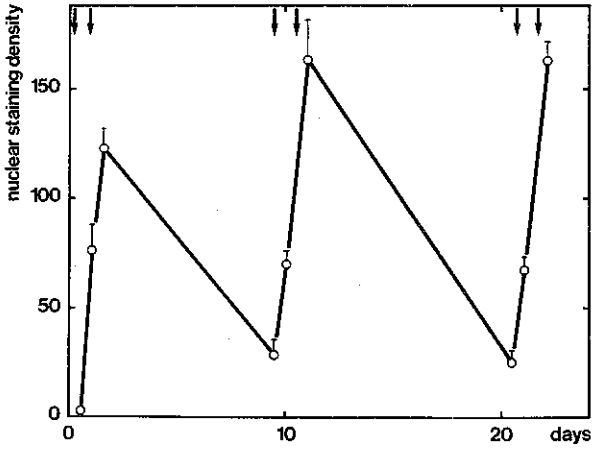

Fig. 7. - Nuclear staining intensity (mean ± standard error) after immunostaining for cDDP-DNA adducts of buccal cells from a cDDP-treated cancer patient (infusions of 50 mg/m^2 cDDP at days 1-2, 10-11, and 21-22) at different times after initiation of the therapy.

Quantification of the immunocytochemical signal

Quantification of the staining intensity is needed, since it will allow more reliable and more pronounced conclusions on the subjects studied. Firstly, evaluation by the eye only can be misleading, since the response of the eye is not linear and integration over stained areas, for example, to compare the total staining of large and small nuclei, is highly unreliable. Secondly, quantification will most likely result in an increased sensitivity. Thirdly, quantification is needed to correct for the fact that nuclei in tissue sections are randomly cut, and thus both adduct and DNA must be measured simultaneously in individual nuclei.

Until now we have concentrated on the quantification of adduct-specific staining. This has been obtained by microdensitometric scanning of the light absorbed by the yellow-brown DAB-derived precipitate, using a recent adaptation of the HIDACSYS system ([32]; Ploem *et al.*, unpublished results). A small, centered spot - 2-5 μm in diameter - is illuminated; the diameter of the measuring diaphragm is about 10 times smaller. This results in a very low level of light scatter and, therefore, in good signal to noise ratios. From the light transmission data obtained by a photomultiplier tube, extinction values are calculated which are a measure for the amount of stain (staining density) in the measured area (pixel). Summation of all measurements obtained for an individual nucleus yields a figure for the amount of damage present. Two examples of quantification will be given.

Aflatoxin-DNA adducts. - Rats were treated with single doses of 0.5, 1.0 or 3.0 mg/kg of the potent hepatocarcinogen aflatoxin B_1 (AFB_1) and 24 h later the liver and a number of other tissues were removed and processed for immunocytochemical staining of DNA adducts. A polyclonal antiserum raised against AFB_1-bovine serum albumin was used. This antiserum has a broad specificity for aflatoxin molecules including binding to AFB_1 - guanine DNA adducts in an ELISA (C.P. Wild, unpublished observations). Positive nuclear staining was restricted to liver, kidney and lung. Fig. 8 shows the data obtained by computer-assisted microdensitometry of the stained liver sections; an almost linear relationship exists between the dose of AFB_1 and the immunocytochemical signal. Data in the literature using [^{3}H]AFB_1 similarly show such a linear relationship with doses of AFB_1 from 1 mg/kg down to 10 ng/kg in rats [33, 34]. Thus the immunocytochemical signal appears to be directly proportional to the adduct level. In further experiments we also used [^{3}H]AFB_1 to treat rats at lower doses (10 μg - 80 μg AFB_1/kg body weight) and quantitated adduct levels in the same samples both by immunocytochemistry in tissue sections, using a high affinity polyclonal antiserum [35] and by radioactivity in isolated DNA. We again observed a linear dose-response by

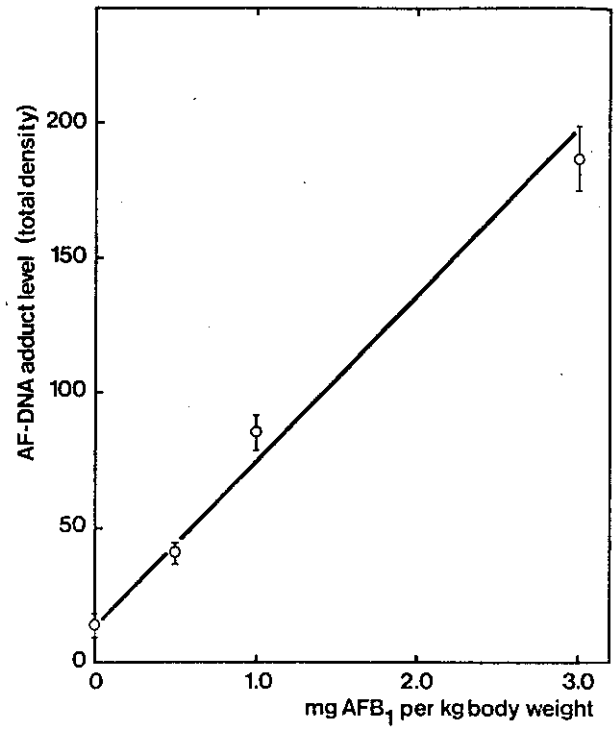

Fig. 8. - Nuclear staining intensity (mean ± standard error) in liver sections from aflatoxin B_1-treated rats (24 h after injection) after immunocytochemical staining for aflatoxin B_1-DNA adducts.

both methods. Using this comparison, a detection limit of 1 adduct in 4 x 10^6 nucleotides or about 2,000 adducts per diploid genome could be calculated. It remains to be established whether this adduct level might be reached in some slowly proliferating, repair deficient cell type in tissues from people exposed environmentally to aflatoxin.

Benzo(a)pyrene DNA adducts. - Using the immunocytochemical technique it recently also became possible to localize Gua-N^2-BPDE in rodent lung tissue after treatment with benzo(a)pyrene (BP) [36, 37]. First a dose-response rela-tionship was established: mice were treated with a single dose of BP (0.5, 1.0, 2.0, 5.0, or 10.0 mg/mice) and killed 24 h later. Lung, liver, heart, stomach and spleen were removed and processed for standard immunocytochemical staining. A polyclonal antiserum, raised against BP diolepoxide-modified DNA was used; the characteristics of this antiserum in an ELISA have been described [38]. In all tissues specific and dose-dependent staining was observed. Fig. 9 illustrates nuclear staining of BP-DNA adducts in the lung. In general, the staining was rather similar for all nuclei within a tissue. This is in agreement with data from studies with radioactively labeled BP, in which a rather homogenous pattern of binding to mouse and rabbit tissues was observed [39]. Quantification of nuclear staining was performed for heart tissue; a clear dose-response relationship was established (Fig. 10). At the highest dose partial saturation of BP binding to DNA seemed to

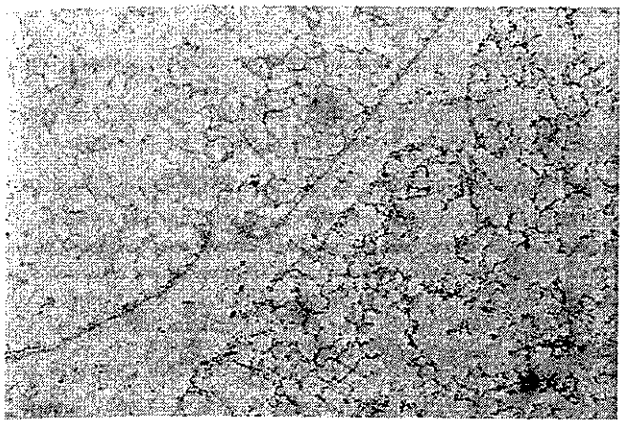

Fig. 9. - Immunocytochemical staining of lung tissue from mice treated with BP. Female BALB/c mice were injected i.p. with 10 mg BP (right) or solvent (left) and lung tissue was collected 24 h later.

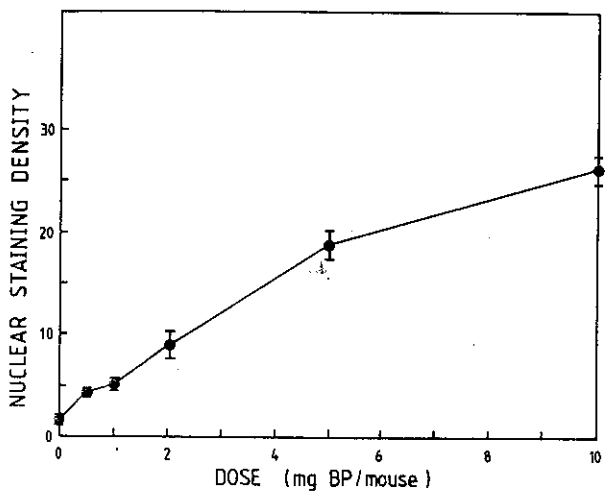

Fig. 10. - Dose-response relationship for BPDE-DNA adducts in the heart of female BALB/c mice. The mice were injected i.p. with 0, 0.5, 1, 2, 5 or 10 mg BP and killed after 24 h.

occur. Adriaenssens *et al.* [40] investigated the binding of BP metabolites to DNA in mouse lung and liver after doses up to 8 mg per mouse. In both tissues the dose-response relationship proved to be sigmoidal; at high doses a saturation of DNA binding became apparent. Our immunocytochemical measurements nicely correlate with these observations, suggesting that the immunocytochemical signal is proportional to the adduct level. Further evaluation of the localization and fate of BP-DNA adducts in different cellular subpopulations should facilitate our understanding of the role played by these adducts in BP-induced tumorigenesis.

Future applications of the immunocytochemical assay for DNA modifications

One of the most promising future applications might well be the determination of the extent and stability of DNA damage in normal and neoplastic cells of cancer patients exposed to DNA modifying, cytostatic drugs. This might lead to assays predicting the response of the tumor and that of other vulnerable tissues to the (intended) treatment, allowing the selection of patients for which the benefits will outweigh the toxic side- effects.

Another potential application of the immunocytochemical analysis of DNA modifications might be the determination of exposure of this target molecule to mutagenic and carcinogenic agents in the environment. This will probably require an increased sensitivity of the immunocytochemical assay by one or two orders of magnitude, although heterogeneity of adduct formation and accumulation of persistent DNA adducts in subpopulations of cells might result in adduct levels sufficiently high to be detected with presently existing methodology.

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