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Title

DETECTION OF DNA DAMAGE IN CELLS EXPOSED TO IONIZING RADIATION BY USE OF ANTI-SINGLE-STRANDED-DNA MONOCLONAL ANTIBODY.

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ABSTRACT

An immunochemical method has been developed for quantitative detection of DNA damage in mammalian cells. The method is based on the binding of a monoclonal antibody to single-stranded DNA. The clone producing this antibody, D1B, was obtained as a by-product from fusion of mouse myeloma cells with spleen cells isolated from a mouse immunized with chemically modified DNA.

The technique is based upon the determination of the percentage single-strandedness resulting from the partial unwinding of cellular DNA under alkaline conditions, a time-dependent process. Single-strand and double-strand DNA breaks, or lesions converted into such breaks in alkaline medium, form initiation points for the unwinding. The extent of unwinding under controlled conditions is a measure, therefore, of the amount of such sites. The method is rapid, does not require radioactive labelling of DNA or physical separation of single- from double-stranded molecules, is sufficiently sensitive to detect damage induced by 1 Gy of ionizing radiation and needs only small amounts of cells. The usefulness of the technique was demonstrated in a study on the induction of damage and its repair in unlabelled cultured Chinese hamster cells and in DNA-containing cells of human blood, both after exposure to ^{60}Co-γ-rays, and in white blood cells and bone marrow cells of X-irradiated mice. A dose-related degree of unwinding was observed and repair could be observed up to 60 min after irradiation.

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SAMENVATTING

Titel: Bepaling van DNA-schade in aan ioniserende straling blootgestelde cellen met behulp van monoklonale antilichamen gericht tegen enkelstrengig DNA

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Ten behoeve van de kwantitatieve detectie van DNA-schade in zoogdiercellen werd een immunochemische bepalingsmethode ontwikkeld. De methode is gebaseerd op de binding van een monoclonaal antilichaam aan enkelstrengig DNA. De cellijn die dit antilichaam produceert, D1B, werd verkregen als een bijprodukt van een fusie van muizemyelomacellen met miltcellen die uit een muis waren geïsoleerd welke was geïmmuniseerd met chemisch-gemodificeerd DNA.

De techniek berust op de bepaling van het percentage enkelstrengigheid die ontstaat bij de gedeeltelijke ontwinding van cellulair DNA onder alkalische condities, hetgeen een tijdsafhankelijk proces is. Enkel- en dubbelstreng DNA-breuken zijn de beginpunten van de ontwinding, alsook lesies die in alkalisch milieu in zulke breuken worden omgezet. Daardoor kan de mate waarin ontwinding, onder gecontroleerde omstandigheden, plaatsvindt worden gebruikt als een maat voor het aantal van dergelijke plaatsen. De methode is snel, vereist geen radioactief merken van DNA of fysische scheiding in enkel- en dubbelstrengige DNA-molekulen, is voldoende gevoelig voor de bepaling van schade die door een dosis van 1 Gy ioniserende straling wordt teweeggebracht en kan worden uitgevoerd met slechts kleine hoeveelheden cellen. De bruikbaarheid van de techniek werd aangetoond in een onderzoek naar de inductie van schade, en het herstel daarvan, in niet-radioactief-gemerkte gekweekte Chinese hamster cellen en in DNA-bevattende cellen in menselijk bloed, beide na blootstelling aan ^{60}Co-γ-straling, en in witte bloedcellen en beenmergcellen van met röntgenstraling bestraalde muizen. De mate van ontwinding vertoonde de verwachte afhankelijkheid van de stralingsdosis en herstel kon worden gevolgd tot 60 minuten na bestraling.

Motivering en toelichting

Bij een aanval met nucleaire wapens is te verwachten dat gewonden moeten worden verzorgd, die tevens een dosis straling hebben ontvangen. Voor het nemen van beslissingen omtrent de toe te passen behandeling is het noodzakelijk te weten, of de ontvangen dosis straling dodelijk is of niet (triage), en zo niet, hoe groot de dosis ongeveer is geweest. Voorts is het van belang de mate van blootstelling te weten, aangezien deze bepalend is voor de afname van de inzetbaarheid van bestraalde manschappen en voor de triage. Op het ogenblik zijn zowel fysische als biologische dosimetrie vereist om aan al deze behoeften van de NATO strijdkrachten tegemoet te komen.

Fysische dosimetrie komt het beste tegemoet aan de behoeften voor het nemen van operationele beslissingen aangaande de inzetbaarheid van de troepen. Daarentegen speelt fysische dosimetrie vrijwel geen enkele rol bij de medische behandeling van bestraald personeel. De behandelende artsen zijn uitsluitend aangewezen op biologische indicatoren, zowel voor de prognose, als voor de te verlenen medische zorg en het volgen van het genezingsproces. Tot nu toe is geen enkele, onder oorlogsomstandigheden bruikbare, biologische indicator gevonden.

De huidige stand van de wetenschap opent echter de mogelijkheid een biologische meetmethode voor de ontvangen stralingsdosis te ontwikkelen. Eén van deze methoden is de immunochemische detectie van stralingsschade in DNA. In het hier beschreven onderzoek kon in de laboratoriumsituatie een gevoeligheid worden bereikt waarmee blootstelling aan 0,5 Gy kan worden aangetoond. Bij de huidige stand van zaken kan de schade binnen vier uur na het nemen van een bloedmonster worden vastgesteld. Een vereiste is wel dat het monster niet later dan een uur na bestraling wordt afgenomen. (Dit laatste houdt verband met het feit dat in de cellen herstelprocessen plaatsvinden die de te detecteren DNA-schade kunnen verwijderen). De methode heeft echter de mogelijkheden tot technische aanpassingen in zich waardoor nog sneller een antwoord kan worden verkregen. Anderzijds zijn verdere ontwikkelingen mogelijk om te komen tot een methode waarmee tot ca 24 uur na de blootstelling nog zinvolle metingen gedaan kunnen worden. Bovendien bestaat de mogelijkheid de methoden toe te passen op enkel-celniveau, zodat ook het vaak noodzakelijke onderscheid tussen partiële en totale lichaamsbestraling kan worden gemaakt. De resultaten van het tot nu toe verrichte ontwikkelingsonderzoek zijn in dit rapport vastgelegd, in de

Inhoudsopgave

INTRODUCTION

There is a wide range of techniques available for detection of damage in cells following exposure to ionizing radiation. Most widely used is the clonogenic assay: irradiated cells are cultured and the fraction that develops into clones is a measure of the reproductive viability of the population. Radiation insult can also be monitored in individual cells by analysis of the chromosomes: breaks in the double helix of the DNA become manifest as various types of chromosome aberrations. Both methods are very time-consuming. Other methods, such as flow cytometry (Milner et al. 1987), are not sensitive enough or require radioactive labelling.

Ahnström and Erixon (1973) demonstrated that double-stranded mammalian DNA in dilute alkali undergoes a time-dependent transformation into the single-stranded form, which process is accelerated upon the introduction of DNA damage by ionizing radiation. These observations provided the basis for the development, by various groups, of sensitive assays of DNA damage, known as DNA-unwinding assays. Rydberg (1975, 1980), using hydroxylapatite columns at elevated temperatures (60°C) to separate single-stranded and double-stranded DNA, established the theoretical background for the quantitation of DNA damage in time-dependent alkaline denaturation procedures. Kohn and coworkers (Kohn and Grimek-Ewig, 1973; Kohn et al. 1974) introduced the alkaline elution of single-stranded DNA through membrane filters, which is based on the principle of increased unwinding after the introduction of single-strand breaks.

Initially, radioactive labelling of the cells had to be applied, to make the quantitative detection of DNA possible. In later studies DNA was quantitated fluorometrically after addition of compounds that fluoresce in the presence of double-stranded DNA (ethidium bromide) or in the presence of both, single- and double-stranded DNA (Hoechst dye H33258). In case of the unwinding assay, application of ethidium bromide allows the direct and selective quantitation of double-stranded DNA (Birnboim and Jevcak, 1981), whereas with H33258 quantitation of DNA damage is based on the differential fluorescence of the dye with double-stranded and single-stranded DNA, respectively (Kanter and Schwartz, 1982). The latter method has the disadvantage of not being very accurate since the difference in fluorescence is

only a factor of two. In neither case physical separation of single- from double-stranded molecules is required, but rather large numbers of cells are needed (about $3x10^6$ per sample). H33258 has been applied also in alkaline elution assays for the quantitation of DNA in the eluate (Stout and Becker, 1982; Murray and Meyn, 1987; Schutte et al. 1988).

Single-stranded DNA can also be detected immunochemically, with specific antibodies. It has been known already for a long time that antibodies can be raised against single-stranded DNA (Halloran and Parker, 1966). Recently, it has been shown that DNA damage can be detected by the use of anti-single-stranded DNA monoclonal antibodies (Frankfurt, 1987).

In the present paper a new immunochemical method is described, in which the unwinding assay is combined with the immunochemical quantitation of the degree of single-strandedness. The essence of the method is, that the very restricted local single-strandedness associated with each (double- and single) strand break is converted into a long stretch of single-stranded DNA by strictly controlled alkaline unwinding. In this way, a very substantial increase has been reached in the sensitivity of the immunochemical detection of single-strand breaks, and also in the assay of DNA damages that can be converted into such breaks.

Several antibodies directed against (modified) DNA are commercially available. The antibodies used in this study for the detection were selected in a standard immunological competition assay. Thermally or alkali-denatured DNA was about 500 times more effective as an inhibitor of antibody-binding than double-stranded DNA. The new method is simple, rapid, inexpensive and only a relatively small amount of cells (about 10^5 per sample) is required. Here, we describe the technique and its application in the direct assay of DNA damage in cultured unlabelled Chinese hamster cells, and in lymphocytes and granulocytes of human blood, all after exposure to ^{60}Co-γ-rays, and in white blood cells and bone marrow cells of X-irradiated mice.

MATERIALS AND METHODS

Cultured Cells

Chinese hamster cells (V79) were grown in monolayer culture in Ham's F10 medium containing 15% new born calf serum (Flow laboratories, Irvine, UK) and antibiotics, as described (Van der Schans et al. 1986). Before the experiment, cells were trypsinized and resuspended in medium. After irradiation and post-irradiation incubation at 37°C, the cells were centrifuged and resuspended in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4 and 15 mM KH_2PO_4, pH 7.4).

Human white blood cells

Venous blood was collected from volunteers in 10-ml evacuated glass tubes containing 15 mg EDTA. After irradiation and post-irradiation incubation at 37°C, the blood was mixed with an equal volume of 0.9% NaCl, and 2.5-ml portions were carefully applied on top of linear density gradients (6 ml; 1.055-1.123 g/ml) of Percoll (Pharmacia, Uppsala, Sweden) in 0.9% NaCl in 10-ml plastic test tubes. After centrifugation for 20 min at $600g_{av}$ (4°C), the clear upper layer containing the plasma, was removed and the lymphocytes, forming a broad band halfway down the gradient, were carefully withdrawn with a pipet. The granulocytes were collected from the opaque band just over the erythrocyte pellet. The cells were freed from Percoll by dilution in PBS and subsequent centrifugation. Finally they were suspended in PBS to a final concentration of about $7x10^5$ cells/ml.

Unfractionated white blood cells were obtained from 0.5 ml venous blood, diluted with an equal volume of 0.9% NaCl, mixed with 4 ml 0.17 M NH_4Cl and kept at 0°C for 20 to 30 min until lysis of erythrocytes was complete. The suspension was centrifuged (5 min, 5°C, centrifugal force 800g at the bottom). The pellet was washed in PBS and resuspended in 1.0 ml PBS.

Murine white blood cells and bone marrow cells

Within 3 min or at appropriate time intervals after the beginning of the irradiation of the mice (BALB/c), about 0.9 ml blood was obtained by heart-punction and collected in 0.5 ml 0.9% NaCl, 8 mM EDTA and cooled on ice. NaCl (0.9%) was added to a final volume of 1.5 ml and 4.5 ml 0.17 M NH_4Cl was added to lyse the erythrocytes (at 0°C). As soon as lysis was

complete (after about 10 min) the white blood cells were centrifuged (5 min 800g, bottom, 4°C), resuspended in 0.9% NaCl and applied on top of linear density gradients as above but with a density from 1.055 to 1.073 g/ml. After centrifugation for 20 min at 700g (bottom, 4°C), the supernatant was removed with a pipet and the pelleted white blood cells resuspended in PBS and centrifuged again at 800g (bottom). Finally the cells were suspended in PBS.

Immediately after the heart punction, the right femur was isolated and further handled on ice. The bone was cut and the marrow cells were flushed out with PBS and directly applied on top of the same type of gradients as indicated above and further handled as the white blood cells.

Irradiations

V79-cells, suspended in medium, and human blood were irradiated in 10-ml plastic test tubes with a ^{60}Co-γ-source (Gamma cell 100, Atomic Energy of Canada Ltd, Ottawa, Canada) at a dose rate of 6 Gy/min. Cells and blood were constantly on ice except for the short period of irradiation.

Mice, placed in a perspex container, were irradiated with a Philips 300 kV X-ray machine (dose rate 4 Gy/min). Beam characteristics were 300 kV; 10 mA; filtration 1.5 mm Cu (corrected), hvl 2.9 mm Cu, mean distance to target 23 cm.

Alkali treatment

Procedures described by Rydberg (1975, 1980) were followed with minor modifications. About 4×10^5 cells suspended in 0.15 ml PBS at 0°C were injected into 0.8 ml "alkaline solution" of different composition, i.e. A: 0.6 M NaCl, B: 1.3 M NaCl, C: 0.6 M NaCl + 0.01 M Na_2HPO_4 , D: 1.3 M NaCl + 0.016 M Na_2HPO_4 + 0.006 M KH_2PO_4, in all cases adjusted with 1 M NaOH to the appropriate pH (12.0 - 12.5) at 20°C and prepared in amounts of 250 ml. The pH was measured with a Radiometer pH-meter, equipped with a B-glass electrode. As a standard a freshly prepared solution of 0.1 M Na_3PO_4 or 0.01 N NaOH was used, which both have a pH of 12.15 at 20°C. The addition of the cells lowered the pH with about 0.1 unit. The mixture was kept in the dark (red light) at 20°C for 6 or 30 min. After cooling on ice for 1 min, an appropriate volume of "neutralizing solution" (0.25 M NaH_2PO_4)

was added; the samples were vigorously shaken and sonicated (Ultrasonics W-370, USA, with microtip, output level 2.5) for 10 sec. After this treatment DNA is in partially denatured form, the extent of denaturation being a function of the number of starting points for unwinding, the size of the unwinding unit and the amount of DNA present. In parallel samples the alkaline lysate was sonicated before neutralization; because of extensive DNA fragmentation by sonication during the unwinding process, in these samples the conversion of native DNA to the single-stranded form is essentially complete. (This has been verified by establishing that additional denaturation for 5 min at 100°C, after neutralization, does not induce more single-strandedness). Then, 0.1 ml of 0.4% sodium dodecyl sulphate (SDS) was added and the samples were stored at -20°C, or, for shorter time periods, at 4°C.

The alkaline solutions A through D were stored under airtight conditions to prevent absorption of CO_2 from the atmosphere. To circumvent small variations in the pH-value, which may result from the adjustment of the pH of freshly prepared alkaline solutions, the same solutions were used several times without further adjustment. When DNA solutions were assayed in a competitive ELISA (see below), they were first heated to 56°C for a few minutes to ensure that the SDS (precipitated at 4°C) would disrupt possible DNA-protein complexes (Rydberg, 1975). Since only about 30 µl are needed per competitive ELISA-assay, the sample volume (1.2 ml) allows several assays.

Monoclonal antibodies

Calfthymus DNA reacted with benzo(a)pyrene(BP)-diolepoxide to an adduct density of 1-2% (Weinstein et al. 1976; Baan et al., 1988), was conjugated with methylated bovine serum albumin (BSA). BALB/c mice were immunized intraperitoneally with 50 µg of this immunogen precipitated on alum, and boostered after 5 weeks with 100 µg without alum. In both cases, Bordetella pertussis bacteria ($2x10^9$) were used as adjuvant. On the third day after the booster injection, spleen cells were fused with SP2/0 plasmacytoma cells by brief consecutive incubations of a mixture of these cells in 41 and 25% polyethyleneglycol (PEG 4000, Merck, Darmstadt, FRG). Hybrid cultures were grown in selective HAT-medium and screened for

specific antibody production in a direct ELISA (see below), with control DNA and BP-modified DNA (1.5% modified) as immobilized antigens. Cells producing specific antibodies were recloned by limiting dilution. Several clones produced antibodies against control DNA without preference for BP-modified DNA. The antibodies from one of these, clone D1B, were used directly from culture supernatant. The immunoglobulin subclass was determined to be IgM. In a competition assay, this antibody was shown to be inhibited by thermally and alkali denatured DNA, but not by native double-stranded DNA, RNA and nucleosides. The antibody is very stable: it can be stored as frozen supernatant for years and there is no noticeable decrease of activity after several cycles of freezing and thawing.

Immunoassays

Enzyme-linked immunosorbent assays (ELISA) were carried out as follows. The wells of microtiter plates (polyvinyl chloride; Costar, Cambridge, Mass., USA; maximum well content 200 µl) were precoated for 16 h at 4°C with poly-L-lysine (Sigma, St. Louis, MO, USA; 1µg/ml in PBS), washed once with PBS, and coated overnight at 37°C and 100% humidity with calf-thymus DNA (1 µg/ml in PBS) which was modified by an oxidative treatment with 0.8% osmiumtetroxide in PBS during 10 min at 50°C (excess osmiumtetroxide was removed by ether extraction). The wells were washed 3 times with 0.05% Tween 20 (polyoxyethylene sorbitan monolaureate; Sigma), then PBS containing 1% fetal calf serum (FCS) was added. Incubation was for 1 h at 37°C, followed by washing with 0.05% Tween 20. Antibody dilutions (direct ELISA) in PBS containing 0.01% SDS and 0.1% FCS, or competition mixtures (competitive ELISA) in PBS containing 0.01% SDS and 0.05% FCS, were put into the wells in duplicate. Incubation was for 45 min at 37°C. The competition mixtures contained various amounts of inhibitor admixed with the appropriate fixed amount of antibody. After 3 washings with 0.05% Tween 20, conjugated second antibody was added (goat anti-mouse-Ig-alkaline phosphatase, Sigma; 1:500 diluted in PBS containing 0.05% Tween 20 and 5% FCS). Incubation was for 45 min at 37°C. After a final wash (3 times with PBS containing 0.05% Tween and once with 0.1 M diethanolamine, pH 9.8), 4-methylumbelliferyl phosphate (Boehringer; 0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$) was added. Incubation was for 1-1.5 h at

37°C. The fluorescence was recorded with a Fluoroskan (Eflab, Finland). [Laboratories that lack fluorescence instruments can use, as an alternative, peroxidase-peroxide conjugates or other chromophores.]

The optimum D1B concentration for the competitive ELISA was determined in a direct ELISA to be a final dilution (in the mixture) of 700 fold (yielding an "uninhibited" fluorescence after 1 h at 37°C of about 30% of the maximum fluorescence detectable with the equipment).

RESULTS

Development of competitive ELISA

For the sensitive, quantitative detection of the degree of alkali-mediated single-strandedness in cellular DNA, we adapted a standard immunological procedure, the enzyme-linked immunosorbent assay (ELISA). The ELISA is based on the binding of antibody molecules to an antigen for which they possess affinity (in our case DNA), that is adsorbed to the wall of minitesttubes (i.e. wells in plastic microtiter plates). Next the bound "first" antibody is assayed via stoechiometric adherence of "second" antibodies carrying an enzyme, the activity of which can be easily tested (e.g. conversion of a substrate into a coloured or a fluorescent product). We used the assay in the so-called competitive mode. In this version, a fixed amount of the "first" antibody is incubated with an unknown quantity of dissolved antigen (the competitor), prior to the transfer to the titration well. In this well, only the excess antibody molecules that have not formed a complex with the competitor will be free to bind to the immobilized antigen attached to the wall. After appropriate washing procedures, the fraction surplus antibody is determined via ELISA ("backtitration"); from this fraction together with the original amount, the quantity of competitor can be calculated.

In our set-up, monoclonal antibodies specific for single-stranded DNA were used, and the wells were coated with (partly) single-stranded DNA. The second antibody was conjugated with alkaline phosphatase, which was assayed via the catalyzed formation of a fluorescent product within a

fixed incubation period. For the determination of the degree of single-strandedness of a certain DNA preparation serial dilutions were made and used as competitor. Furthermore, the fluorescence measured in the absence of competitor DNA ("infinite dilution") was established (100% signal; no competition), as well as the signal when all antibody molecules had been complexed during the pre-incubation (100% competition). By intrapolation, that dilution of the DNA preparation was determined which should give a signal just half-way between these two values (the "50%-inhibition point"). Comparison of this dilution with the 50%-inhibition point obtained with the same preparation after it had been made completely single-stranded, finally yielded the percentage single-strandedness.

In practice, several problems had to be solved. In order to obtain a sufficiently reproducible immunochemical assay for the detection of the amount of single-stranded DNA in a mixture of single-stranded and double-stranded DNA. certain precautions had to be taken. For a reproducible coating of the wells of the microtiter plates, a precoating with poly-L-lysine appeared to be necessary. Furthermore, modification of the single-stranded coating-DNA was required in order to decrease the affinity of the D1B antibodies for the coating material, thus preventing transfer of D1B-antibodies originally bound to the competitor DNA, to the immobilized antigen. Several modifications were tried; superior results were obtained when double-stranded calfthymus DNA was used that had been subjected to an oxidative treatment with osmiumtetroxide and which was used for coating without further denaturation. An additional important prerequisite was to carry out the washing of the plates with water containing 0.05% Tween 20, both before and after addition of the first antibody. This washing step probably ensures that only firmly bound (coating) DNA remains attached to the wall. Washings following the second antibody addition had to be carried out with solutions of the same ionic strength as that of the antibody solution added. The final essential step appeared to be the addition of SDS to the DNA solution, followed by heating to 56°C, just before dilution for the competitive assay. SDS is a detergent known to disrupt DNA-protein linkages (Noll and Stutz, 1968).

Application of these experimental conditions in the competitive immuno-assay resulted in "ideal" competition curves (Fig. 1).

Alkali treatment

It was our aim to establish conditions for a reproducible, controlled partial unwinding of DNA in alkaline medium, to such an extent that each single-strand break would yield a single-stranded region of substantial length. To this end, several variations in pH, ionic strength and buffer concentrations were tested. Table 1 presents a summary of a number of variations applied, and their effect on the degree of radiation-induced single-strandedness (due to unwinding at single- and double-strand breaks) in DNA of mammalian cells from different origins, both unirradiated and after irradiation with γ-rays (5 Gy).

The results indicate that under the conditions applied, controlled partial unwinding can be obtained in the pH range 11.8-12.2. At pH's below 11.8 very little single-strandedness resulted, and no difference was seen between irradiated and control cells, just as when the alkaline treatment had been omitted. Obviously, hardly any unwinding is induced under these conditions. Above pH 12.2, unwinding proceeds too fast, to the extent that only a slight difference remains between irradiated and unirradiated cells. As a rule, the percentage of single-strandedness increases with ionic strength, phosphate concentration and (most critically) the final pH, in DNA of both unirradiated and irradiated cells. An important observation is that the rate of unwinding does not appear to vary with the cell type: in logarithmically growing V79-cells it is not significantly different from that in resting human peripheral blood cells. The best conditions for the alkaline treatment, i.e. those resulting in the largest relative difference in single-strandedness between irradiated and unirradiated cells, appeared to be a low phosphate concentration (or no phosphate at all) and a final pH of 12.0. The period of treatment may be 6 as well as 30 min. For good reproducibility, the final pH should be well standardized.

An important step in the procedure is the sonication, which is performed immediately after neutralization to break down the DNA and thus to prevent reannealing of the single-strand regions. When the mixture was not sonicated immediately, but e.g. at 10 min after neutralization, or not at all, much less irradiation-dependent single-strandedness could be detected.

The results shown were obtained at 20°C; lower temperatures resulted in a lower degree of unwinding. Furthermore, it appeared to be useful to cool the samples just before neutralization; this resulted in a decrease of a-specific single-strandedness.

Induction and repair of "single-strandedness" in γ-irradiated V79-cells

The assay developed was used to analyse the induction of damage responsible for the irradiation-dependent alkali-mediated single-strandedness in V79-cells exposed to various doses ^{60}Co-γ-rays. An example of the results of such an experiment is shown in Fig. 1. It is clear that exposure to a radiation dose as low as 1 Gy is well detectable.

To calculate the percentage single-strandedness in a particular sample, the dilution of that sample resulting in 50% inhibition of antibody-binding was compared to the dilution giving 50% inhibition after the sample had been made completely single-stranded. The percentage single-strandedness thus obtained was found to be a linear function of irradiation dose within the range applied (Fig. 2).

Removal of damage leading to the detected single-strandedness, during an incubation of the irradiated cells at 37°C, was followed over a period of up to 60 min. In agreement with data obtained with the unwinding method of Ahnström and Erixon (1973) and with the alkaline elution method (Kohn et al. 1974), a fast removal was observed, followed by a much slower decrease (Fig. 2). Nevertheless, a significant amount of single-strandedness is still detectable at 60 min after irradiation at a dose of 5 Gy.

Induction and repair of single-strandedness in lymphocytes and granulocytes in irradiated human blood

Also with lymphocytes and granulocytes isolated from human blood after exposure to ^{60}Co-γ-rays, linear dose-effect relations were obtained with respect to the damage detected in this immuno-assay (Fig. 3a). The slope for the granulocytes is less steep than that for the lymphocytes. Also removal was studied (Fig. 3b). After 60 min a substantial part (15 and 35% in lymphocytes and granulocytes, respectively) of the initially detected alkali-induced single-strandedness is still detectable. A dose-

dependent damage-induction could also be obtained in the total, unfractionated sample of lymphocytes and granulocytes which was isolated after lysis of the erythrocytes in total human blood by treatment with ammonium chloride and subsequent washing (data not shown).

Induction and repair in white blood cells and bone marrow cells of irradiated mice

The method was also applied on cells isolated from X-irradiated mice. Due to the isolation procedure, some time elapsed between the irradiation and the assay. The amount of single-strandedness determined for the white blood cells 3 min after the onset of irradiation was higher than the value found for bone marrow cells at 4 min (Fig. 3c). The values are in the same range as those obtained with the human blood cells. The data shown in figure 3d represent the levels of persisting (detected) single-strandedness measured in each tissue at different times after sacrificing, normalized to the value of the sample obtained as soon as possible after irradiation. In both, white blood cells and bone marrow cells, at 60 min after the onset of irradiation of the mice with a dose of 5 Gy, still a substantial level of radiation-induced single-strandedness was detectable.

DISCUSSION

Quantitative determination of lesions in DNA after exposure to ionizing radiation forms an essential step in the examination of the sequence of events leading to mutation of the DNA or, eventually, to the death of the cell. With the availability of the new immunochemical method described in this paper, quantitation of damage is possible even at the low dose ranges suitable for the evaluation of the effects of ionizing radiation and other mutagens with respect to various biological endpoints (e.g. survival of mammalian cells; Arlett and Harcourt, 1980, Van der Schans et al. 1986).

An important feature of the method is its high sensitivity, which makes it possible to carry out the assay with a very small number of cells. This is due to the fact that the radiation-induced single-stranded-

ness can be amplified considerably by the mild denaturation procedures applied on the irradiated cells. Every break is a starting point for unwinding of the DNA. Under our conditions, this unwinding proceeds over a long distance (in the order of $5x10^5$ nucleotide pairs). This is based on the assumption that a dose of 5 Gy induces $12.5x10^{-10}$ single-strand breaks per dalton DNA (Van der Schans et al. 1983). This corresponds to about one single-strand break per 10^6 nucleotide pairs. When about 50% of the DNA is single-stranded, as is the case in DNA of V79-cells, after a dose of 5 Gy and a 30-min treatment at pH 12.0 in "solution C" (Table 1), then one single-strand break results in about 10^6 nucleotides being present as single-stranded DNA. Even with antibodies like those used in this work, which have not a very high affinity for single-stranded DNA (detection limit approx. 10^{-13} mol/assay, on nucleotide basis), it appeared to be possible to detect levels of radiation-induced lesions as low as 10^{-19} mol per assay. In these experiments the number of cells treated with alkali was about $3x10^5$, of which only a minor proportion is needed for the ELISA.

It is reasonable to assume that the alkali-treatment of the cells can be carried out on a still smaller scale, so that assays with a total of about $2x10^4$ cells will be feasible. With regard to the applicability of the method, it should be realized that it requires high molecular weight DNA. It cannot be applied, therefore, on DNA isolated from cells or tissues, in view of the break induction unavoidably occurring during isolation. Up to the moment of alkaline unwinding, the integrity of the DNA should be maintained as much as possible. The lower detection limit of radiation dose is comparable to those reported for other methods (alkaline elution, DNA unwinding) but, as mentioned in the Introduction, these need radioactively labelled cells, or are rather laborious and require large amounts of cells.

An important question still to be answered refers to the nature of the lesions determined with the immunochemical assay. In this respect the repair studies contribute some interesting information. With all cell types tested, at 60 min after irradiation a considerable fraction of the single-strandedness initially measured is still detectable. Although differences appear to exist between various cells with regard to dose-depen-

dency of the induction of the lesions involved, in absolute numbers the results at 60 min after 5 Gy are very similar. The relatively high level of residual DNA lesions measured with the new method contrasts with the very low level of single-strand breaks as detected with alkaline elution (Van der Schans et al., 1983), although both methods are based on the principle of unwinding in alkaline medium. Evidently, the new method detects certain relatively persistent lesions that are not measured in the alkaline elution assay. This observation suggests that the new method might be useful as biological indicator for a rapid estimate of radiation dose to which persons accidentally have been exposed.

It would be an important further improvement if some other persistent base damages could be first converted, inside the cell, into single-strand breaks or alkali-labile sites. This conversion might be induced by treatment of permeabilized cells with damage-specific glycosylases, damage-recognizing endonucleases (as present in Micrococcus luteus extracts), or by chemical conversion into alkali-labile sites or strand breaks.

The pH at which conversion to single-strandedness occurred was in the same range as reported by Rydberg (1975, 1980), but was much lower than the value (pH 12.8) applied by Birnboim and Jevcak (1981). An explanation might be that the pH-adjustment, which is very critical for the final result, is rather tricky in this pH-region. In the case of Birnboim and Jevcak's experiments also the presence of (high concentrations of) other solutes (0.25 M meso-inositol-1 mM $MgCl_2$-3 M urea-0.8 mM cyclohexanediamine tetraacetate) during the alkali-treatment might be of influence.

The amount of radiation-induced single-strandedness determined in DNA samples of logarithmically growing V79-cells was not significantly different from that of non-dividing human lymphocytes (Table 1). This is in disagreement with the observation of Rydberg (1975) that cells in S-phase (which holds for most of the V79-cells) show a faster strand separation than cells at other stages of the cell cycle.

Because the immunochemical method described in this paper is simple, rapid and sensitive, it may have application in several areas related to human health. In addition, the fact that such low numbers of cells are required per assay, makes it possible in principle to detect single-strandedness in any mammalian cell type that can be prepared as a homogeneous

suspension. Especially when attempts are successful to apply this technique also on DNA at the single-cell level, then the research aims described above will become attainable within the next years.

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Table 1. Summary of the effects of various alkali-treatment conditions on the relative amounts of single-strandedness in irradiated cells

cells	alk. solution	final pH	treatment time (min)	% single-stranded DNA after 0 Gy	ratio SS after 5 and 0 Gy
lym	C	11.8	6	1.0	6.0
lym	C	12.0	6	1.0	18.0
lym	C	12.1	6	5.0	4.0
lym	C	12.0	6	1.1	12
lym	D	12.0	6	0.9	3
lym	D	12.2	6	5.0	5.0
lym	D	12.4	6	40	1.5
gran	D	12.0	6	0.9	1.7
gran	D	12.2	6	10	2.5
gran	D	12.4	6	45	1.2
h wbc	D	12.2	6	11	4.5
lym	C	12.0	30	4	12
m wbc	C	12.0	30	9.5	9
bm	C	12.0	30	9	4.3
lym	A	12.0	30	1.6	5.5
lym	B	12.0	30	3	7.3
lym	C	12.0	30	6	13
lym	D	12.0	30	6.5	4.6
lym	D	12.0	30	1	18
lym	D	12.2	30	15	3.3
lym	D	12.4	30	50	1.4
gran	D	12.0	30	2.7	7
gran	D	12.2	30	22	2.2
gran	D	12.4	30	50	1.2
h wbc	A	12.0	30	4	4
h wbc	B	12.0	30	12	3.3
h wbc	D	12.0	30	26	1.6
h wbc	D	12.0	30	10	4.7
h wbc	D	12.2	30	50	1.6

About 4×10^5 cells suspended in 0.15 ml PBS at 0°C were injected into 0.8 ml "alkaline solution" of different composition, i.e. A: 0.6 M NaCl, B: 1.3 M NaCl, C: 0.6 M NaCl + 0.01 M Na_2HPO_4, D: 1.3 M NaCl + 0.016 M Na_2HPO_4 + 0.006 M KH_2PO_4, each adjusted with 1 M NaOH to the appropriate pH (between 11.9 and 12.5). The addition of the cells in PBS lowered the pH by 0.09-0.11 units. The mixture was kept in the dark (red light) at 20°C for 6 or 30 min. An appropriate volume of "neutralizing solution" (0.25 M NaH_2PO_4) was added, and the samples were vigorously shaken and sonicated. Then, the degree of single-strandedness was assayed in the competitive ELISA. lym: human lymphocytes; gran: human granulocytes; h.wbc, m wbc: white blood cells obtained after lysis of erythrocytes in human, resp. mouse blood, followed by Percoll-gradient centrifugation; bm: bone marrow cells of the mouse; ratio SS: single-strandedness in DNA of cells irradiated with 5 Gy of ^{60}Co-γ-rays relative to that of unirradiated cells.

different cell-preparations.

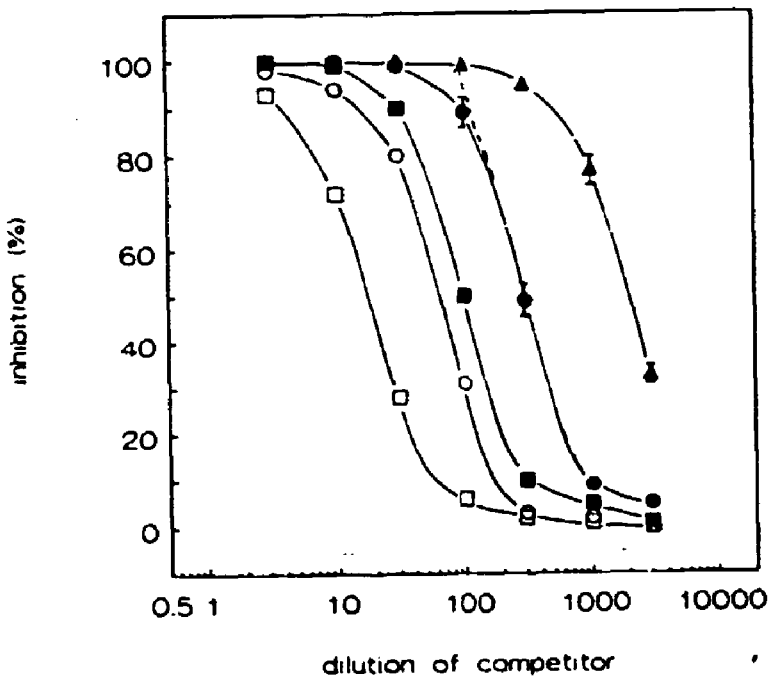
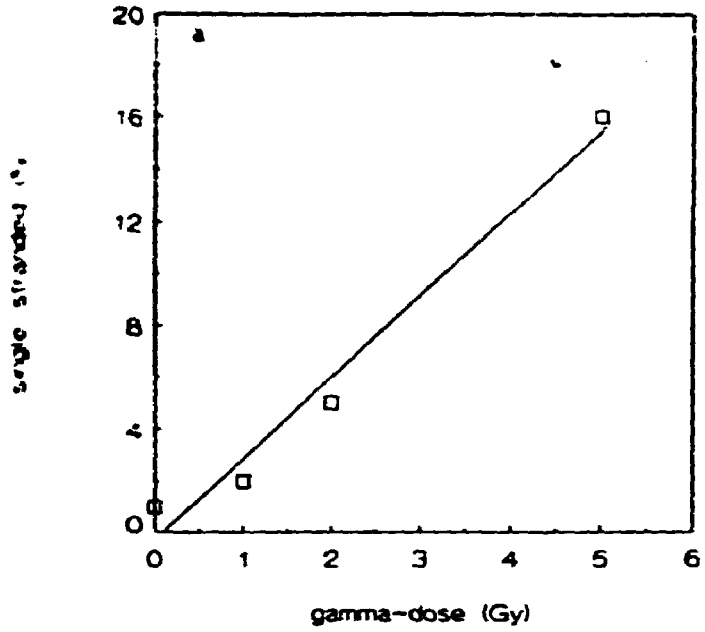


Fig. 1
An example of a series of competition curves of DNA of V79-cells, exposed to various doses of ^{60}Co-γ-rays at 0°C and kept at pH 12.0 for 6 min (in "alkaline solution C", see Table 1). □: unirradiated; O: 1 Gy; ■: 2 Gy; ●: 5 Gy. ▲: unirradiated cells, the DNA of which was completely denatured. A fixed amount of D1B antibody was mixed with various dilutions of competitor DNA, and the percentages remaining free (= uninhibited) were determined in an ELISA. Determinations of each dilution of competitor DNA were carried out in duplicate. The vertical bars represent the range between duplicates and are shown only when larger than the symbols. The broken curve represents the shape of the curve expected if all free D1B antibody binds to the wall with an infinitely high affinity, superimposed on the 2 Gy curve.



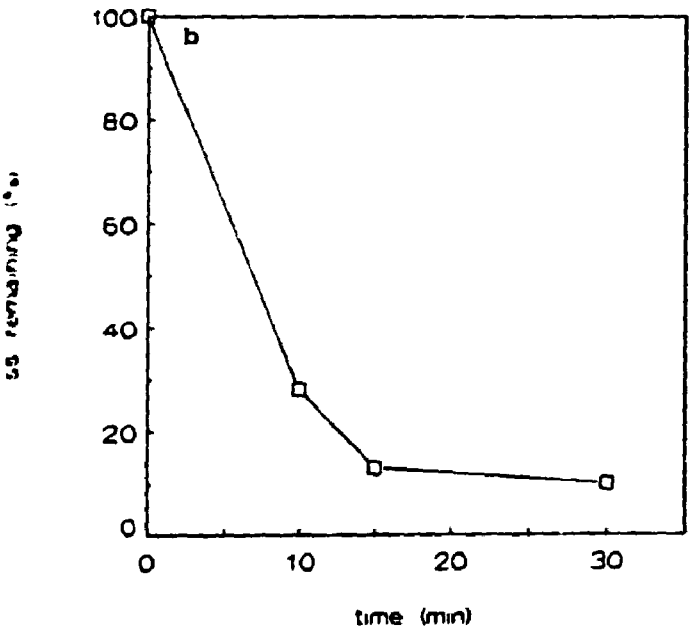


Fig. 2
Induction and removal of damage in V79-cells, exposed to ^{60}Co-γ-rays at 0°C. Panel a: dose-response curve of DNA single-strandedness induction, derived from the competition curves of Fig. 1. panel b: removal of sites giving rise to single-strandedness in alkali (ss) when V79-cells, exposed to a dose of 5 Gy, were incubated in suspension at 37°C for various periods of time before the treatment with alkali (solution C, pH 12.0, for 6 min). The percentage single-stranded DNA was determined as in "Materials and Methods". Each determination was carried out in duplicate. The mean values are presented, which contain an estimated error of about 10%.

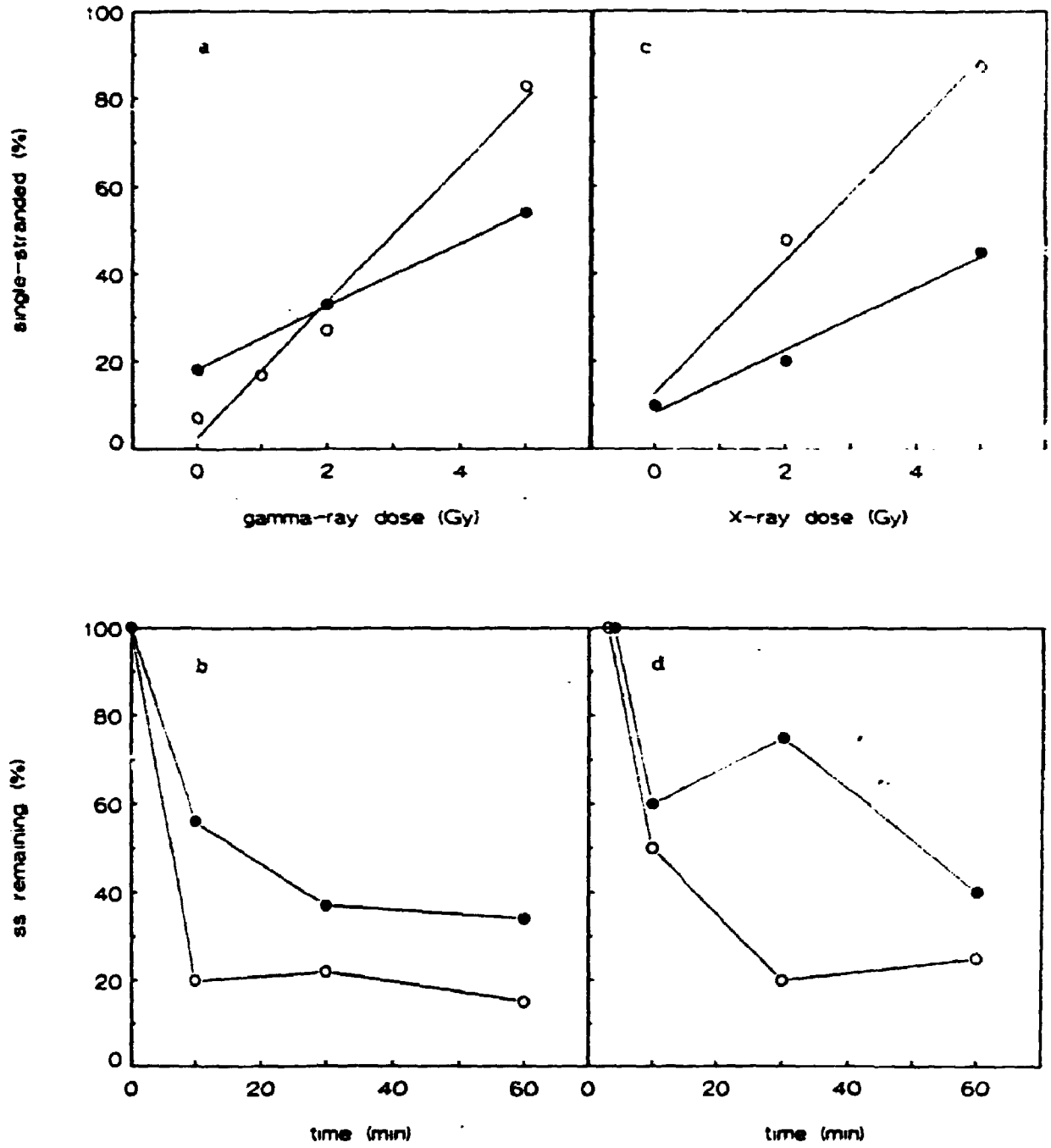


Fig. 3
Induction and removal of sites in DNA giving rise to single-strandedness in alkali. Panel a and b: human lymphocytes (O) and granulocytes (●) in blood exposed to ^{60}Co-γ-rays at 0°C. Panel c and d: white blood cells (O) and bone marrow cells (●) in X-irradiated mice. Panel a and c: dose-response curves of DNA single-strandedness induction. Panel b and d: removal of single-strandedness. Human blood was irradiated with a dose of 5 Gy and subsequently incubated at 37°C for various periods of time. Mice were irradiated with an X-ray dose of 5 Gy and at the indicated times after the onset of irradiation, blood and bone marrow cells were collected. The single-strandedness measured at the shortest possible time interval after the onset of radiation was set at 100% (for white blood cells about 3 min and for bone marrow cells 4 min). For the single-strandedness assay, cells were treated with alkaline solution C at a final pH 12.0 for 30 min.

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