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MICRONUCLEI

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INTRODUCTION

What Are Micronuclei?

Micronuclei consist of small amounts of DNA that arise in the cytoplasm when chromatid/chromosomal fragments or whole chromosomes are not incorporated into daughter nuclei during mitosis, often because these fragments do not possess a centromere. Acentric fragments remain behind at anaphase, whereas chromosomal elements with centromeres are drawn toward the spindle poles (Schmid, 1975). The fragments of DNA left behind are incorporated into secondary nuclei. These, much smaller than the main nucleus of the cell, are known as micronuclei. Hence, the formation of micronuclei requires a dividing cell population. Micronuclei are about 1/20 to 1/5 the size of the main nucleus. Usually there is only one micronucleus formed per cell (Jenssen, 1982). The frequency of micronuclei is usually reported as the number of cells containing micronuclei per total cells counted.

Micronuclei may be formed as the result of agents that cause chromosome breaks (clastogens) or as the result of agents that cause damage to the spindle apparatus (Jenssen, 1982). Damage to DNA, which ultimately leads to the formation of micronuclei, may occur during various parts of the cell cycle and may result from exposure to a variety of environmental agents (see Chapter 8 for a review of the cell cycle). Methyl methanesulfonate (MMS) induces micronuclei during DNA synthesis, alkylating agents during the G_1 and DNA synthesis phases, and agents that operate on the spindle apparatus induce micronuclei during mitosis (Jenssen, 1982). Clastogenic agents, such as x-rays, often cause micronuclei to form by creating chromatid or chromosomal fragments, whereas agents that cause spindle dysfunction can lead to the formation of micronuclei from loss of whole chromosomes. Micronuclei that result from loss of whole chromosomes tend to be larger than those that result from chromosome breaks (Högstedt and Karlsson, 1985).

What Is Their Significance?

It is generally believed that any agent capable of causing structural damage to DNA is also potentially carcinogenic, as most carcinogens have been found to be mutagens (McCann and Ames, 1976). DNA damage can lead to oncogene amplification and transposition, mechanisms thought to be involved in the neoplastic transformation of cells (Stich, 1986).

The presence of micronuclei in a population of cells indicates chromosome damage that has occurred as the result of exposure to a variety of genotoxic agents that either cause chromosome breaks or spindle dysfunction. The frequency of micronuclei alone, however, cannot distinguish between these two types of damage. Thus, the frequency of micronuclei in the cells of a tissue serves as a marker of exposure and of biological response to genotoxic agents. An important characteristic of micronuclei is that they reflect damage that has occurred in the presence of the host's metabolic activation and detoxifying mechanisms.

The formation of micronuclei and the incidence of chromosome breakage or loss are believed to correlate in dividing cell populations (Heddle et al., 1983). In animal studies, micronuclei are associated with standard cytogenetic indices of chromosomal breakage (Goetz et al., 1975). Micronuclei are much easier and faster to score (Heddle, 1973), however, and are more sensitive indicators of chromosome damage than chromosome aberrations (Jensen and Hüttel, 1976; Jensen and Nyfors, 1979; Högstedt et al., 1981b). An increase in the incidence of micronuclei is associated with an increase in chromosome or chromatid aberrations (Jenssen, 1982). As noted previously, chromosomal aberrations are believed to be early steps in carcinogenesis, although there is no documented association between the frequency of chromosomal aberrations and the development of cancer.

History of the Micronucleus Assay

Micronuclei were originally discovered in red blood cells by Howell in 1891 and later characterized by Jolly in 1905. As a result, micronuclei in red blood cells are often referred to as Howell–Jolly bodies (Jenssen, 1982). Investigators have known for at least 50 years that micronuclei form in association with exposure to radiation. The first major attempt at using micronuclei as indicators of cytogenetic damage, however, was not done until fairly recently when Evans and co-workers (1959) exposed plant root tips to radiation (Heddle et al., 1983). In 1966, Schroeder (1966) showed that micronuclei occurred in bone marrow cells after cytogenetic damage by chemical mutagens. Schmid and co-workers (Boller and Schmid, 1970) and Heddle (1973) tried to determine which characteristics of bone marrow cells would best indicate cytogenetic damage in vivo. They concluded that the presence of micronuclei in polychromatic erythrocytes (PCEs) served as a particularly useful indicator of cytogenetic damage in mice (Heddle et al., 1983). Schmid and co-workers (Boller and Schmid, 1970) and Heddle (1973) independently devised a micronucleus test with mouse PCEs to detect chromosomal damage caused by genotoxic chemicals as a relatively quick and inexpensive method of screening for potential carcinogens.

Currently, there is no one micronucleus assay. Such tests can be performed on a variety of species and with different types of cells; the techniques differ depending on the cell type assayed. The assays have been performed most often with PCEs from

mouse bone marrow. Bone marrow sampling, however, is highly invasive. Although the bone marrow assay has been used in occupational settings (Högstedt et al., 1981a), it is unlikely to be widely used in human populations. The bone marrow assay in humans may, therefore, be limited to hospitalized patients who have bone marrow samples taken for diagnostic or therapeutic purposes. For example, Högstedt et al. (1981b) explored the use of micronuclei in bone marrow cells as a prognostic indicator of survival among patients with acute nonlymphocytic leukemia. In general, however, such populations are unlikely to be representative of the groups to which one would like to make inferences. Because of its limited application for human studies, the bone marrow assay is not discussed further.

Micronucleus assays in humans have also been carried out using peripheral red blood cells (Schlegel et al., 1986), peripheral blood lymphocytes (Countryman and Heddle, 1976; Högstedt et al., 1983b; Pincu et al., 1984; Norman et al., 1978; 1985; Fenech and Morley, 1985, 1986; Högstedt and Karlsson, 1985; Kormos and Köteles, 1988), and exfoliated cells such as buccal mucosa cells and urinary tract cells (Stich et al., 1982, 1984, 1985; Stich and Rosin, 1983a,b; Stich and Rosin, 1984; Rosin and German, 1985; Fontham et al., 1986). The following section will review methods of performing these assays with various cell types and explore issues relating to their usefulness in epidemiologic research.

THE MICRONUCLEUS ASSAY AND PERIPHERAL BLOOD ERYTHROCYTES

Schlegel et al. (1986) studied the utility of the micronucleus assay in detecting genotoxic effects of chemical agents in peripheral blood erythrocytes (PBEs). PBEs are derived from erythroblasts, which are erthrocyte stem cells located in the bone marrow. Micronuclei are formed during the last mitosis leading to the development of PBEs. They remain in the PBEs even after the nucleus is expelled. Although perhaps not target cells of interest, PBEs may serve as indicators of exposure to genotoxic agents.

Assay Techniques (Schlegel et al., 1986)

1. Draw a sample of peripheral blood.
2. Apply to microscope slide.
3. Air dry smear.
4. Fix for 5 minutes in absolute methanol.
5. Stain with Wright's stain (for total erythrocytes) or acridine orange (for reticulocytes). (For acridine orange, immerse slides for 5 minutes in pH 7.4 sodium phosphate buffer (1% by weight) containing 0.02 mg acridine orange per milliliter. Rinse for 10 minutes in pH 7.4 phosphate buffer.)
6. Wet mount slides.
7. Examine by fluorescent microscopy using Zeiss fluorescein isocyanate filter.
8. Score micronuclei by hand at X 1000 magnification under oil in 2000 total erythrocytes per person.

Methodologic Issues

The chief obstacle in the use of peripheral blood erythrocytes is the fact that in healthy individuals the spleen removes micronucleated red blood cells from circula-

tion. People with normally functioning spleens have very low frequencies of micronuclei (0/100,000 cells), even after treatment with a known clastogenic chemotherapy agent (Schlegel et al., 1986).

Schlegel et al. (1986) studied the effects of genotoxic agents on the frequency of micronuclei among splenectomized and nonsplenectomized individuals. Among 12 people with intact spleens who had not been exposed to radiation or chemotherapy, the frequency of micronuclei in PBEs was 0/56,000 cells analyzed. The frequency of micronuclei in PBEs of 16 people with intact spleens who had received at least 1 month of chemotherapy during the 4-month period before sampling was 0/100,000 cells. The authors note that one can detect an increased frequency of micronuclei in nonsplenectomized individuals receiving chemotherapy, if sampling is done shortly after administration of the drug. Because there is rapid removal by the spleen of micronucleated erythrocytes, the efficiency of the spleen will affect micronucleus frequencies.

Among splenectomized individuals who had not received chemotherapy, micronuclei began to appear in PBEs shortly after splenectomy. The frequency of micronuclei reached a steady state of 4/2,000 PBEs after 4 months. After chemotherapy, these people experienced an increase in frequency of micronuclei, with a higher frequency corresponding to increased duration of treatment. Those undergoing chemotherapy ultimately had micronucleus frequencies five times higher than splenectomized control levels. It was noted that micronuclei persisted in the PBEs of splenectomized individuals for about 120 days (the life span of an erythrocyte). About 4 months after the end of chemotherapy, the frequency of micronuclei returned to baseline levels.

If the exposure is not continuous, scoring total erythrocytes may not provide the information sought. Because many erythrocytes will have been derived from erythroblasts not exposed to the genotoxic agent, the assay would underestimate the effect of the exposure. The sensitivity of the assay could be improved by only evaluating newly formed reticulocytes that were derived from exposed erythroblasts in the bone marrow. Erythroblasts mature and give rise to circulating reticulocytes after about 3 to 4 days. Because reticulocytes are believed to appear in the blood within 3 to 4 days and disappear within 4 to 10 days, sampling would have to take place within 4 to 10 days after exposure to the agent. If total erythrocytes are scored, the sensitivity decreases but there is less of a chance of missing the point of highest elevation in micronucleus frequency. The other alternative is to examine micronucleus frequencies in both total erythrocytes and in reticulocytes (Schlegel et al., 1986).

Another methodologic consideration is that people who are folate deficient (even mildly so) have elevated levels of micronuclei in their peripheral blood erythrocytes (Everson et al., 1988). Therefore, investigators should control for folate levels in epidemiologic studies using the micronucleus assay with PBEs.

Summary of Advantages and Disadvantages of The Micronucleus Assay Using Peripheral Blood Erythrocytes

Advantages

1. The sampling technique for this assay, the drawing of peripheral blood, is relatively noninvasive.

2. The assay is fairly inexpensive and easy to perform.

3. A dose–response relationship has been noted between exposure (duration of

chemotherapy treatment) and frequency of micronuclei among splenectomized individuals (Schlegel et al., 1986).

4. The cessation of treatment leads to a decrease in micronucleus frequencies among splenectomized individuals (Schlegel et al., 1986).

Disadvantages

The major disadvantage of using the micronucleus assay with peripheral blood erythrocytes is that the spleen removes micronucleated erythrocytes from circulating blood. Thus, people with normally functioning spleens often do not have detectable levels of micronuclei. The efficiency of the spleen will also affect micronucleus frequencies. The use of this assay is, therefore, most likely limited to splenectomized subjects.

THE MICRONUCLEUS ASSAY AND PERIPHERAL BLOOD LYMPHOCYTES

Several investigators have explored the use of the micronucleus assay with peripheral blood lymphocytes (PBLs) (Countryman and Heddle, 1976; Norman et al., 1978, 1985; Högstedt et al., 1983b; Pincu et al., 1984; Fenech and Morley, 1985, 1986; Kormos and Köteles, 1988). Like PCEs and PBEs, lymphocytes may not necessarily be the target cell population of interest with respect to future development of disease, but the frequency of micronuclei in PBLs may serve as an in vivo index of exposure to genotoxic agents. As with PBEs, the sampling method involves drawing peripheral blood, a fairly noninvasive technique.

Methodologic Issues

Performing micronucleus assays with circulating blood lymphocytes presents a unique set of problems. In essence, the dilemma is that, although circulating lymphocytes are not a naturally dividing cell population, cell division is necessary to induce micronuclei. Inducing lymphocytes to divide in cell culture overcomes this limitation, allowing investigators to use an in vitro assay to detect genotoxic damage that occurs in vivo. But, because one cannot be certain that cell culture techniques do not themselves induce micronuclei, it is important to perform the micronucleus assay with control cells to quantify background frequencies induced by the technique.

Methods for using peripheral blood lymphocytes in micronucleus assays are currently under development. At least four different methods of preparing lymphocytes for the assay have been tested. Several investigators have scored micronuclei in lymphocytes after hypotonic treatment with potassium chloride. This method has been used to show elevated frequencies of micronuclei among patients after angiocardiography (Norman et al., 1978) and among smokers (Högstedt et al., 1983a), but it has drawbacks. The hypotonic treatment destroys the lymphocyte cytoplasm making detection of micronuclei difficult; micronuclei may also be indistinguishable from cellular debris or may become separated from the cell of origin (Högstedt, 1984).

Högstedt (1984) proposed a method of preparing lymphocytes that preserves the cytoplasm. This method offers a more precise determination of micronucleus frequencies. It has been used to detect elevated levels of micronuclei among 38 workers

exposed to low levels of styrene compared with 20 controls (p=.005), controlling for age and smoking status (Högstedt et al., 1983b).

One of the major issues in developing the micronucleus assay has been determining which lymphocytes should serve as the denominator of the micronucleus frequency. Only cells that have divided at least once after exposure are competent to have formed micronuclei, and should be included in the denominator. Pincu et al. (1984) have developed a staining procedure that identifies which lymphocytes have undergone cell division. Therefore, with this technique one can determine which lymphocytes could not have developed micronuclei, improving scoring speed, and reducing the assay's variability due to differences in cell proliferation. Both x-rays (Countryman and Heddle, 1976) and certain chemicals, including bromodeoxyuridine (BrdUrd) (Boyes and Koval, 1985), used in lymphocyte cell cultures, slow cell division, delaying the time of the first mitosis and leading to an underestimate of micronucleus frequency if cells that have not divided are included in the denominator.

Still, knowing that the lymphocytes have divided does not answer the question of how many cell divisions they have undergone. Ideally, one would like to identify cells that have undergone only one cell division. The more cell divisions after the time of exposure, the more dilute the micronucleus frequencies become. This is more of an issue with acute, as opposed to chronic, exposures.

Fenech and Morley (1985, 1986) have developed the most promising method of identifying cells that have divided only once. To do this, they used a mitogen, phytohemagglutinin (PHA), to stimulate lymphocytes in culture to divide, then allowed the cells to complete nuclear division, and used cytochalasin B to block the completion of cell division. It is then possible to score micronuclei in the binucleate cytokinesis-blocked cells. Cytochalasin B was not found to cause chromosome abnormalities or induce micronuclei when cells were cultured for 48 to 72 hours at a concentration of 3.0 μg/ml. Fenech and Morley made no mention of the ability of PHA to induce micronuclei. The frequency of micronuclei found with this assay depends on the proportion of cells that are induced by the mitogen to divide (Fenech and Morley, 1985). Lymphocytes from different individuals vary in their response to PHA.

Assay Techniques for the Fenech and Morley (1985) Lymphocyte Assay

1. Draw a sample of peripheral blood from the subject.
2. Separate peripheral blood lymphocytes from whole blood on Ficoll–Hypaque gradients, wash twice in Hanks' balanced salt solution, and resuspend in McCoy's modified medium 5A containing 15 percent heat inactivated fetal calf serum.
3. Culture lymphocytes in 0.2-ml microwells at a concentration of 0.5×10^6 cells/ml.
4. Add PHA (5 μg/ml, Burroughs Wellcome reagent grade).
5. Culture cells at 37°C in a humidified atmosphere containing 10 percent CO_2.
6. Prepare cytochalasin B as a stock solution in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/ml divided into small portions and stored at −70°C.
7. Thaw solution and dilute in saline.
8. Add cytochalasin B at a concentration of 3.0 μg/ml to culture 44 hours after the start of the culture.
9. Stop culture at 72 hours.

10. Centrifuge cells and prepare slides.
11. Score a minimum of 800 binucleate cells per individual (or score until 45 micronuclei are observed (Fenech and Morley, 1986)).
12. Score slides with X1000 magnification.

Specimen Sampling Schedule

Because lymphocytes circulate throughout the body, they can reflect exposures that occur at various sites. Only damage that has occurred during the life of the lymphocyte can be detected. Damage should be detectable almost immediately, perhaps within a day of an individual's exposure to the genotoxic agent (time to appearance of the marker).

The life span of lymphocytes varies from about 3 days to 20 years (Leavell and Thorup, 1976), depending on the type of lymphocyte (persistence of the marker). Ninety percent of lymphocytes have a half-life of about 3 years. The half-life of the remaining 10 percent is 1 to 10 days. Approximately 50 percent of all lymphocytes and most peripheral lymphocytes circulate between the blood, spleen, lymph nodes, and other tissues. Most of these lymphocytes are of the long-lived T-cell type. The turnover rate of all lymphocytes in the body is about 2 to 5 percent per day. Because lymphocytes have a fairly long life span and relatively low repair rates, damage can accumulate over the years (Natarajan and Obe, 1982). With acute exposures, sampling should occur as close as possible to the time of exposure, as lymphocyte turnover will eventually dilute micronucleus frequencies. Sampling should probably be done at various intervals so as not to miss peak elevations in micronucleus frequencies. For chronic exposures, determining sampling time is less of an issue. Lymphocytes of chronically exposed people, however, have heightened repair mechanisms (Natarajan and Obe, 1982).

Detecting Dose–Response Relationships

Several studies have noted dose–response relationships between lymphocyte exposure to radiation in culture (Pincu et al., 1984; Fenech and Morley, 1985, 1986; Kormos and Köteles, 1988) and the frequency of micronuclei. At doses above 400 rads, Pincu et al. (1984) found a decreased micronucleus frequency, probably from cell death. Erexson et al. (1987) noted a dose–response relationship with in vivo exposure of mice to the chemotherapeutic agent diaziquone, using the Fenech and Morley method to analyze micronucleus frequency. Högstedt et al. (1983b), however, found no dose–response relationship between levels of styrene exposure and frequency of micronuclei. Perhaps the range of styrene exposures (1–36 ppm time weighted average) was not sufficient to show an effect.

Assay Variability

There are two main sources of variability in lymphocyte assays. The length of time cells are cultured is one source of technique-induced variation in some lymphocyte assays. Högstedt et al. (1983b) found no statistically significant difference in micronucleus frequencies after exposure to styrene when they cultured cells for 72 hours; they did, however, find a significant difference when they cultured cells for 96 hours. Peak micronucleus frequencies occurred between 80 and 88 hours. The second source of variability arises from the observers who score the assay. Ideally, one observer who

is blind to exposure status should score smears for both exposed and unexposed subjects. Högstedt et al. (1984) had two different technicians score cultures for micronuclei. Although there was a good correlation between the results of the two observers (r=0.95), there was a 30 percent difference in micronucleus frequencies.

Potential Confounders

Several investigators using different methods for the lymphocyte assay have found an increase in micronucleus frequency with increasing age (Högstedt, 1984; Norman et al., 1985; Fenech and Morley, 1986). Högstedt (1984) reported an increase in the frequency of micronuclei in association with smoking and Norman et al. (1985) noted a nonsignificant increase in micronuclei among women as compared with men.

Summary of Advantages and Disadvantages of the Micronucleus Assay Using Peripheral Blood Lymphocytes

Advantages

1. Lymphocyte sampling can be done in a fairly noninvasive manner.
2. Chromosome damage that has occurred in vivo in the presence of the host's activating and deactivating mechanisms can be assayed in vitro.
3. Because lymphocytes circulate throughout the body, exposures at various sites can be detected in the peripheral blood.
4. Because lymphocytes have a fairly long life span and relatively low repair rates, damage can accumulate over the years (Natarajan and Obe, 1982).
5. Dose–response relationships have been noted with the lymphocyte assay (Pincu et al., 1984; Fenech and Morley, 1985, 1986; Erexson et al., 1987; Kormos and Köteles, 1988).
6. The Fenech and Morley (1985) assay allows easy recognition of binucleate cells and, hence, easy scoring for micronuclei. The assay offers enhanced statistical power: (1) because of the low spontaneous frequency of micronuclei, 4.4±2.6 micronuclei/500 cytokinesis-blocked cells, and (2) because one can score twice the number of micronuclei for a given effort in these binucleate cells (Fenech and Morley, 1985).
7. The lymphocyte assay is more rapid, less expensive, less labor-intensive, and more sensitive than techniques for analyzing chromosome aberrations (Fenech and Morley, 1986).
8. Lymphocytes can be stored in liquid nitrogen for future use.

Disadvantages

1. Lymphocytes may or may not be target cells of interest with respect to future cancer development.
2. Culture conditions, such as length of time in culture, may affect micronucleus frequencies (Högstedt et al., 1983b).
3. Culture conditions can affect the proportion of cells that divide, and therefore, the eligibility of cells to develop micronuclei. To minimize the variability of the assay, investigators should keep culture conditions as consistent as possible.

THE MICRONUCLEUS ASSAY AND EXFOLIATED CELLS

Many investigators, in particular, Stich and co-workers (Stich et al., 1982, 1984, 1985; Stich and Rosin, 1983a,b) have explored the benefits of performing the micronucleus assay in various exfoliated cell populations. Exfoliated cells are typically epithelial cells sloughed from the surface of a body cavity, such as the respiratory tract, the gastrointestinal tract, and the genitourinary tract. Because epithelial cells are derived from basal cells, damage induced by a genotoxic agent at the basal cell layer should be reflected in the frequency of micronuclei in exfoliated cells. The presence of micronuclei in exfoliated cells serves as an internal dosimeter (Stich and Rosin, 1983a), measuring the extent to which an environmental agent is associated with DNA damage to tissues in vivo and, hence, potentially providing a measure of the risk of cancer development.

Exfoliated cells have several positive features in the micronucleus assay: most of the cell populations can be sampled noninvasively; because they are naturally dividing cell populations, cell culture is not necessary; and they allow assessment of in vivo damage to such target cells of interest as cells of the mouth, nasopharynx, esophagus, stomach (Stich et al., 1983), colon (Heddle et al., 1982; Goldberg et al., 1983), bladder (Reali et al., 1987), lungs (Fontham et al., 1986), and cervix (Fontham et al., 1986; Rosin et al., 1987).

Assay Techniques

The most extensively studied exfoliated cells in micronucleus assays have been buccal mucosa cells. A description of one preparation technique for buccal mucosa smears follows (Stich et al., 1982).

1. Rinse the person's mouth to remove particles of food and other debris.
2. Scrape the buccal mucosa with a wooden tongue depressor.
3. Apply cells to microscope slide.
4. Within 24 hours, pretreat air-dried mucosa cell smears for 3 minutes with 50 percent glycerin.
5. Fix smears with ethanol/glacial acetic acid (3:1) and air dry again.
6. Stain with Feulgen reaction.
   a). pretreat in 1 N HCl for 2 minutes at room temperature and 6 minutes at 60°C
   b). transfer into 1 N HCl at room temperature for 2 minutes
   c). rinse in distilled water
   d). put into Schiff reagent for 90 minutes
   e). wash with three changes (2 minutes each) of freshly prepared sulfite solution
   f). rinse twice with running water
7. Counterstain for 5 to 10 seconds with fast green dissolved in 95 percent ethanol.
8. Mount dehydrated preparations (tertiary butanol, xylene) in permount.
9. Count micronuclei under light microscope.

Methodologic Issues

Much more work has been done in human populations with the micronucleus assay in exfoliated cells than with other cell types. To explore the use of the micronucleus assay in epidemiologic studies, the following sections discuss such relevant issues as when to sample exfoliated cells, the assay's ability to detect dose–response relationships, and its variability and cost. Epidemiologic field studies that employed the assay illustrate the discussion.

Specimen Sampling Schedule

In deciding when to sample exfoliated cells after an individual is exposed to a potentially genotoxic agent, one must take two factors into consideration: the length of time it takes for cells at the basal layer to migrate to the surface (time to appearance of the marker) and the length of time between exposure and complete sloughing of all potentially exposed cells (persistence of the marker). For acute exposures, the rate at which cells migrate to the surface affects the frequency of micronuclei (Stich and Rosin, 1984). Information relevant to the time course of cell migration from the basal cell layer to exfoliation can be derived from a study conducted by Stich et al. (1983) in which patients received radiation therapy. Patients who received radiotherapy to the head and pelvic regions experienced an increase in frequency of micronuclei among buccal mucosa cells and urinary tract cells, respectively. After radiation therapy ended, micronuclei began to disappear after 5 to 7 days and completely disappeared after 24 days. Five to 7 days is believed to be the time necessary for undamaged new cells to migrate from the basal cell layer to the surface of the epithelial cell lining (Stich et al., 1983). For chronic exposures, sampling can take place at any time. Sampling for acute exposures should take place between 5 and 24 days after exposure to the agent of interest as it takes about 5 to 7 days for new cells to arrive at the surface of the lumen and 24 days for all affected cells to be completely sloughed.

Depending on the the type of tissue, the exposure, and the severity of damage to the tissue caused by the exposure, one may have to alter sampling times. For example, cell turnover rates differ from one tissue to another. Because radiation slows mitosis (Countryman and Heddle, 1976), exposure to radiation may cause a delay in the migration of cells from the basal layer to exfoliation. On the other hand, injury to a tissue may increase cell proliferation rates as the tissue attempts to repair the damage.

Detecting Dose–Response Relationships

Several studies (Stich et al., 1982; Stich and Rosin, 1983a,b; Fontham et al., 1986; Reali et al., 1987; Sarto et al., 1987) provide evidence for the assay's ability to distinguish dose–response relationships. In a study performed in India, Stich et al. (1982) compared the frequency of micronuclei in buccal mucosa cells among three groups. Two groups chewed betel quids, a known risk factor for oral squamous cell carcinoma (IARC, 1985), and the third group (the controls) did not. The results of the study showed that chewers had significantly higher frequencies of micronuclei in their buccal mucosa cells than nonchewers ($p<.001$). There was also a higher frequency of micronuclei at the site where the quid touched the mucosa. Right-sided chewers had higher frequencies of micronuclei on the right side and left-sided chewers had higher frequencies on the left. Those who chewed on both sides showed no difference in

frequency of micronuclei on one side of the mouth or the other. The investigators did not find a clear dose–response relationship between number of quids chewed per day and the frequency of micronuclei, but noted a significantly higher frequency of micronucleated cells among those who chewed more than 15 quids per day (average 7.05% micronucleated cells) compared with those who chewed less than four quids per day (average 1.50% micronucleated cells) (Stich et al., 1983).

Several other conclusions can be drawn from this study: (1) the assay detects recent, rather than cumulative damage over time, as the number of micronucleated cells did not increase with number of years the subject chewed, a variable often employed as a surrogate for dose; (2) the micronucleus assay reflects differences in seemingly similar exposures to genotoxic agents, as those who chewed betel quids consisting mainly of perfumed tobacco, dried betel nut, betel leaf, lime, and spices had a higher frequency of micronuclei than those who ate raw betel nuts with betel leaves and lime; and (3) the micronucleus assay does not indicate stage of disease, as leukoplakia cells showed no elevation in frequency of micronuclei (Stich et al., 1982). Leukoplakia, however, may be caused by factors other than those leading to the formation of micronuclei. Even if micronuclei are present, rapid proliferation of leukoplakia cells may make them nondetectable.

Stich and Rosin (1983b) investigated the separate and combined effects of cigarette smoking and alcohol consumption on the frequency of micronuclei in buccal mucosa cells. They sampled 500 cells per person. Only one exposure category showed a significant increase in micronucleus frequencies: the group of people who both consumed 150 ml of alcohol or more per day and smoked at least a pack of cigarettes per day. Smoking and alcohol together have been shown in epidemiologic studies to synergistically increase the risk of oral cancer (Schmidt and Popham, 1981). Stich and Rosin found a dose–response relationship, based primarily on the number of cigarettes smoked per day, in the group that used both alcohol and cigarettes. They found an eightfold increase in the number of micronucleated cells among those who consumed alcohol and smoked three or more packs of cigarettes per day and a 4.2-fold increase among those who smoked one to two packs per day. Smoking three packs of cigarettes per day without drinking or drinking 1.2 L of alcohol per day without smoking did not yield a detectable increase in frequency of micronuclei in buccal mucosa cells.

In contrast, Fontham et al. (1986) did not find a dose–response relationship between number of cigarettes smoked and the frequency of micronuclei in buccal mucosa cells, bladder, cervical, or bronchial cells. Nor did they find a synergistic increase in micronucleus frequencies in people who both smoked cigarettes and drank alcohol. In their study of 486 patients at Charity Hospital in New Orleans, however, they did find elevated frequencies of micronuclei in all four organ sites among smokers (of any amount) in comparison with nonsmokers. They saw no significant elevations in micronucleus frequencies among nonsmokers married to smokers. It is possible, the authors suggest, that there was too little heterogeneity in numbers of cigarettes smoked in this population to reveal a dose–response relationship.

Similar findings were reported by Reali et al. (1987) who did not find a correlation between numbers of cigarettes smoked and frequency of micronuclei in urothelial cells of 12 smokers and 12 nonsmokers. Small sample size and lack of heterogeneity in numbers of cigarettes smoked may account for these results.

Both Stich and Rosin (1983a) and Sarto and co-workers (1987) have noted in-

creases in micronucleus frequencies in exfoliated cells upon exposure to increasing doses of radiation. At high doses, however, micronucleus frequencies decrease due to cell death.

Intervention Studies

Stich et al. (1984, 1985; Stich, 1987) and Muñoz et al. (1987) explored the utility of the micronucleus assay in assessing the efficacy of chemopreventive measures. Stich and co-workers (1984) supplemented the diet of 40 rural Filipino betel nut chewers with vitamin pills containing retinol (100,000 IU/week) and β-carotene (300,000 IU/week) twice weekly for three months. Eleven chewers who were not supplemented served as controls. β-carotene, a precursor of vitamin A (retinol), inhibits the action of tumor promoters and acts as a free radical scavenger. Retinol is important in maintaining the integrity of epithelial cells. The investigators sampled 300 buccal mucosa cells per person, both before and after the vitamin supplementation. The results of the study confirmed the hypothesis that retinol and β-carotene supplements decrease micronucleus frequency in buccal mucosa cells. Supplemented subjects experienced a threefold decrease in the mean proportion of cells with micronuclei over the 3-month period (4.2–1.4%). The 11 nonsupplemented betel quid chewers experienced no decrease (4.3–4.8%). The spontaneous frequency among 17 nonchewing controls was 0.0 to 0.8 percent initially and 3 months later.

Thus, Stich et al. first showed that exposure to a mutagen increased the frequency of micronuclei (Stich et al., 1982) and then showed that exposure to a protective agent lowered the frequency of micronuclei. They found similar results when they supplemented the diets of Inuit snuff dippers with β-carotene (Stich et al., 1985). Supplementation, however, was not successful in reducing the number of micronucleated cells of the mucosa of the palate and tongue of inverted smokers (Stich, 1987). (Inverted smokers put the burning end of the cigar in their mouths.)

Muñoz et al. (1987) noted mixed results in a study of micronucleus frequency in exfoliated cells from the buccal mucosa and esophagus among citizens of the People's Republic of China known to have an elevated risk of esophageal cancer. They studied 610 subjects who were randomly assigned to receive either an active treatment (15 mg or 50,000 IU of retinol, 200 mg of riboflavin, and 50 mg of zinc) or a placebo once a week for 13.5 months. They saw no statistically significant difference in micronucleus frequencies in buccal mucosa cells after treatment. They did, however, observe a significant reduction in micronucleus frequencies in cells of the esophageal mucosa ($p=.04$).

Assay Variability

Several studies illustrate issues related to the variability of micronucleus assays in exfoliated cells (Stich et al., 1982; Rosin and German, 1985; Sarto et al., 1987). Rosin and German (1985) investigated micronucleus frequencies in exfoliated cells of people with Bloom's syndrome, a chromosome breakage syndrome that is associated with an increased risk of cancer. They found that people with Bloom's syndrome had significantly higher frequencies of micronuclei in buccal mucosa and urinary tract cells than heterozygotes or controls. Samples taken from eight sites within the same persons showed that people with this genetic syndrome have low intraindividual variability in micronucleus frequencies.

Both the vulnerability of particular tissues to a genotoxic agent and the presence of other genotoxic exposures will affect intraindividual variability. Variability across tissue sites will be high for site-specific exposures such as chewing tobacco on the right or left side of one's mouth, as Stich et al. (1982) suggested earlier. Therefore, in epidemiologic studies of such exposures one must carefully select the sampling sites.

Interindividual variability depends largely on the activation and detoxifying mechanisms of both the person and the tissue under investigation. Two individuals may receive the same exposure to an agent, but because of differences in metabolism, may differ in the frequency of micronuclei present; therefore, the biologically effective dose may be different. Stich and Dunn (in press) note that background frequencies of micronuclei in buccal cells of nonsmokers vary relatively little, whereas the bronchial cells of nonsmokers who have no known exposures to polycyclic aromatic hydrocarbons show a wide range of frequencies of micronuclei.

Differences in criteria for scoring micronuclei is another source of assay variability. In fact, differences in criteria have resulted in different laboratories reporting different background frequencies of micronuclei in buccal mucosa cells (Sarto et al., 1987). One difficulty in scoring micronuclei is that some exposures, for example, snuff, cause severe cytotoxicity. As exposed cells degenerate, fragments of DNA from the nucleus move into the cytoplasm where they resemble micronuclei, that is, these fragments result from cytotoxic rather than genotoxic mechanisms. To obtain more accurate micronucleus counts, Sarto and co-workers (1987) suggest excluding cells with degenerative characteristics and analyzing at least 3000 cells per subject.

Potential Confounders

Because the micronucleus assay is a nonspecific indicator of chromosome breakage and spindle dysfunction, increased frequencies of micronuclei appear after exposure to any chemical or agent that causes such damage. Viruses, chemicals, x-rays, and tobacco can elevate micronucleus frequencies (Stich and Rosin, 1983b; Stich and Rosin, 1984; Fontham et al., 1986).

Cost

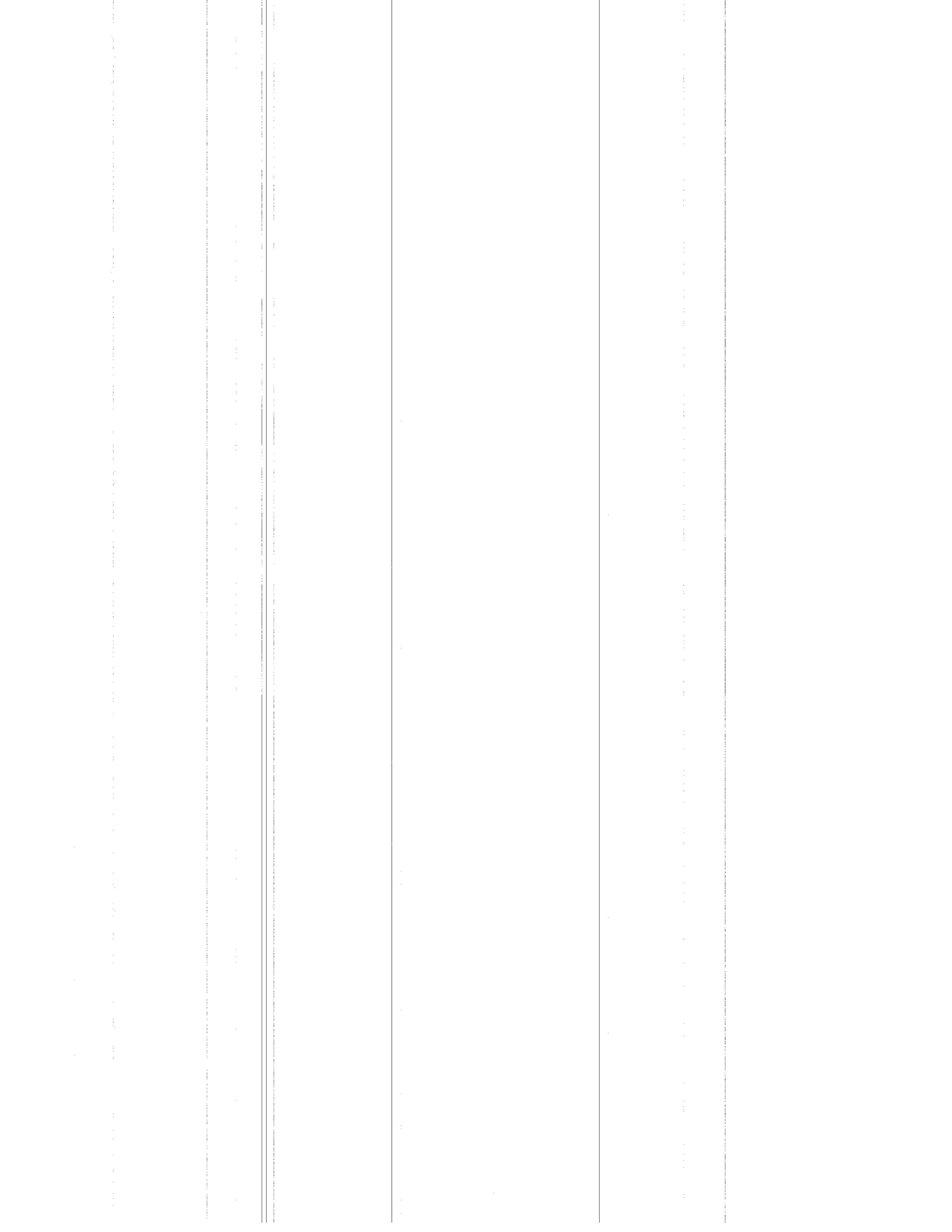
The micronucleus assay in exfoliated cells is a relatively inexpensive and rapid assay. Stich and Rosin (1983b) note that an experienced cytotechnologist can examine 500 cells in 40 to 60 minutes. Eventually, automated scoring of micronuclei will reduce costs (Callisen et al., 1986; Fenech et al., 1988); Stich and Dunn (in press) claim that a microscope image processing scanner can be used to scan 10^5 cells per minute.

Summary of Advantages and Disadvantages of the Micronucleus Assay Using Exfoliated Cells

Advantages

1. The frequency of micronuclei in exfoliated cells provides evidence of a biological response to a genotoxic agent in a naturally dividing cell population.

2. The assay has successfully identified people at high risk of cancer: betel quid chewers (Stich et al., 1982), people who both smoke and drink alcohol (Stich and Rosin, 1983b), and snuff dippers (Stich et al., 1985).



3. The assay allows the detection of damage in vivo to a whole organism that is using its activating and deactivating metabolic machinery.

4. One can detect evidence of exposure to a genotoxic agent in a target cell population likely to develop cancerous lesions (Stich et al., 1982).

5. One can sample a variety of sites and cell types in a minimally invasive or noninvasive manner. Such sites include the buccal mucosa (cheek cells), bronchi (sputum cells), urinary bladder (cells in urine), nasopharynx, esophagus, cervix, and colon.

6. The low spontaneous frequency of micronuclei in buccal cells (0.0–0.8%) enhances the statistical power of the assay (Stich et al., 1984).

7. The study of cigarette smoking and alcohol consumption (Stich and Rosin, 1983a) suggests that the assay detects the damaging effects of two or more agents acting together.

8. The test can be applied to large groups of people.

9. Prepared buccal cell smears can be stored indefinitely for future use. Fixed tissues, such as biopsy specimens, can be evaluated for micronuclei (Stich, 1987).

10. The test is quick and inexpensive. An experienced cytolotechnologist can examine 500 cells in 40 to 60 minutes (Stich and Rosin, 1983b).

11. The scoring of micronuclei (percentage of micronucleated cells, number of micronuclei per cell, and amount of DNA per micronucleus) can be automated (Stich and Dunn, in press; Callisen et al., 1986; Fenech et al., 1988).

Disadvantages

1. The assay cannot identify cytogenetic damage by agents that neither break chromosomes nor damage the spindle apparatus. Based on micronucleus frequencies alone, one cannot distinguish between damage induced by clastogenic agents and damage induced by agents causing chromosomes to lag at anaphase (Heddle et al., 1983).

2. It is difficult to know whether the frequency of micronuclei in exfoliated cells is representative of damage induced in the basal layer. At high doses of radiation, for example, a decrease in micronucleus frequency may result from cell killing (Stich and Rosin, 1983a).

3. It is possible to miss very recent, short-term, or past exposures (Stich, 1986).

THE USE OF THE MICRONUCLEUS ASSAY IN EPIDEMIOLOGIC RESEARCH

Invasiveness

To be useful in large scale epidemiologic research, an assay should possess a low degree of invasiveness. Bone marrow sampling, for example, is highly invasive. For large populations, it is far more practical to use less invasive techniques, such as drawing blood, to obtain peripheral blood erythrocytes (PBEs) and peripheral blood lymphocytes (PBLs), and obtaining scrapings of exfoliated cells.

Specimen Sampling Schedule

The issue of when to sample cells in relation to the time of exposure depends both on the cells to be sampled and on the biology of the tissue from which they are derived.

One must keep in mind that micronuclei indicate recent damage, not the effects of cumulative damage from chronic exposures. For many of these assays, multiple sampling times will guard against missing peak or intermittent elevations in micronucleus frequencies. With chronic exposures, sampling time is less of an issue.

For peripheral blood erythrocytes, time to appearance of micronuclei is based on the time between exposure and the appearance of new erythrocytes in the blood, which is believed to be about 3 to 4 days (Schlegel et al., 1986). Because erythrocytes persist in the blood for about 120 days, sampling should be done 4 to 120 days after exposure. In individuals with normally functioning spleens, however, the spleen removes micronucleated cells from the circulation. Thus, this assay is likely to be limited to people who have been splenectomized.

In lymphocytes, damage should be detectable almost immediately (within a day). Lymphocytes vary in longevity from 3 days to over 20 years (Leavell and Thorup, 1976) and 90 percent are long-lived, with a half-life of about 3 years (Natarajan and Obe, 1982). Because lymphocyte turnover will dilute micronucleus frequencies, sampling should occur as soon as possible after exposure.

In exfoliated cells, the time to appearance of micronuclei depends on how long cells take to migrate from the basal layer, where the damage occurs, to the surface of the epithelium where they are exfoliated. In buccal cells, this is believed to be about 5 to 7 days. After about 24 days all potentially affected cells are sloughed (Stich et al., 1983). Therefore, sampling should occur between 5 and 24 days after exposure.

Detecting Dose–Response Relationships

Ideally, one would like to see a clear dose–response pattern between the frequency of micronuclei and the dose of a particular agent to quantitate the degree of exposure. Dose–response patterns depend on the cell type assayed as well as on the genotoxic agent (Schmid, 1976). Among splenectomized individuals, a dose–response relationship was noted between duration of chemotherapy treatment and frequency of micronuclei detected in peripheral blood erythrocytes (Schlegel et al., 1986). In studies of exfoliated buccal cells, Stich and Rosin (1983b) noted a dose–response relationship, based primarily on the number of cigarettes smoked per day, among people who both smoked cigarettes and drank alcohol. Fontham et al. (1984), on the other hand, did not find a dose–response relationship in buccal, bladder, or bronchial cells in association with numbers of cigarettes smoked, nor did Stich et al. (1982) find a clear dose–response relationship between the frequency of micronuclei and the number of betel quids chewed per day. They did, however, note a significantly higher frequency of micronucleated cells among those who chewed more than 15 quids per days versus those who chewed less than four quids per day (Stich and Rosin, 1983a). Decreased micronucleus frequencies have been observed in populations at high risk of cancer who were given vitamin supplements containing β-carotene (Stich et al.,1984, 1985; Muñoz et al., 1987).

Sensitivity, Specificity, and Positive Predictive Value

The micronucleus assay has been evaluated for sensitivity and specificity only in mouse bone marrow studies. Jenssen and Ramel (1980) evaluated the micronucleus

assay as a short-term test for detecting carcinogenic damage. They compared the sensitivity and specificity of the mouse micronucleus assay and the Ames test against the known carcinogenic potential of a variety of chemicals, based on malignant tumor induction studies in experimental animals and epidemiologic studies in humans.

They analyzed 143 chemicals evaluated by the micronucleus assay and 115 assayed by the Ames test. Both the micronucleus assay and the Ames test had a specificity of about 80 percent. The two tests differed significantly with respect to sensitivity. The Ames assay had a sensitivity of 80 percent, whereas the micronucleus assay had a sensitivity of 58 percent. Together, the two tests had a sensitivity of 86 percent. Jenssen and Ramel (1980) suggest that increasing the number of cells sampled from 1,000 to 4,000 per animal would increase the sensitivity of the micronucleus assay. Studies of chromosome damage in *Drosophila*, however, indicate that the dose required to induce point mutations is lower than the dose needed to produce nondisjunctional events or chromosome aberrations. Thus, the sensitivity of the micronucleus assay may never reach that of the Ames test (Jenssen and Ramel, 1980).

From a literature search of mouse micronucleus assays performed on various chemicals, Heddle et al. (1983) determined that of 150 known clastogens tested, 50 percent produced an increased incidence of micronuclei. The 50 percent sensitivity in clastogen detection agrees fairly well with the 58 percent sensitivity found by Jenssen and Ramel (1980) in carcinogen detection.

Given this information, how should one interpret the results of the micronucleus assay? The interpretation of a positive result depends on the assay's specificity and the prevalence of micronuclei in the population under investigation. The positive predictive value of the assay depends on the prevalence of micronuclei in the population. Although the assay has a high specificity, the sensitivity is low. If the prevalence of micronuclei in the population is also low then the false-positive rate will be high lowering the positive predictive value of the assay. Given the assay's low sensitivity, a negative test may be interpreted several ways. It may be that the agent really does not cause DNA damage. On the other hand, the agent may cause DNA damage that is not detected for a variety of reasons. For example, the assay may not be sensitive enough to detect damage at low doses. Or, the power of the test may be too low. Power depends in part on the number of cells scored per subject and the number of subjects tested. Another explanation for a negative result may be that the agent does not cause DNA damage to the cells assayed, but does damage other target cells, such as those in the liver. Or again, the agent may cause damage that cannot be detected by the micronucleus assay.

Thus, the assay may not be a good discriminator of individual exposure, but by detecting population elevations in micronucleus frequencies, may be useful in distinguishing populations likely to develop cancer. Sensitivity and specificity estimates from human studies do not exist. Therefore, the implications of the mouse bone marrow studies with regard to humans are unclear.

Assay Variability and Intraindividual and Interindividual Variability

The variability of the assays depends on both the laboratory procedures used and the characteristics of the individuals assayed. In both the mouse bone marrow assay and the exfoliated cell assay, the test seems to yield reproducible results (Schmid, 1976;

Rosin and German, 1985). Control levels of micronuclei are consistently low in mice (Schmid, 1976) and in buccal mucosa cells (Stich et al., 1984), although different investigators report different baseline levels of micronuclei in exfoliated cells (Sarto et al., 1987). Establishing criteria for the identification of micronuclei, adequate training of personnel, running both positive and negative controls, and having the same individual—one who is blind to exposure status—read slides from both exposed and control subjects can reduce differential misclassification bias from laboratory errors.

Intraindividual variability depends both on the exposure of interest and the cell type assayed. Certain chemicals have an affinity for certain tissues, and within tissues, there may be site-specific elevations in micronucleus frequencies. If the same person is assayed over time, cell turnover and additional exposures may affect micronucleus frequencies. Interindividual variability is largely influenced by the metabolic functioning of the individuals assayed. The same dose of the same agent may elicit different responses from different individuals.

Cost, Time, Personnel

The micronucleus assay is a relatively quick, simple, and inexpensive way to detect chromosomal damage. Compared with classic methods of assessing chromosome aberrations, such as metaphase scoring, the micronucleus assay is less expensive to perform and an order of magnitude faster to score (Heddle, 1973). Because micronuclei are easily recognizable, the assay does not require highly trained personnel. The test has enhanced statistical power because a large number of cells are available and can be scored in a short period of time. Stich and Dunn (in press) claim that the assay lends itself to automated scoring; a microscope image processing scanner can scan 10^5 cells per minute.

Potential Confounders

In human studies, potential confounders of the effects of chromosome-damaging agents on micronucleus frequencies include age (Högstedt, 1984; Norman et al., 1985; Fenech and Morley, 1986), with the frequency of micronuclei increasing with advancing age, cigarette smoking (Högstedt et al. 1983a; Högstedt, 1984; Fontham et al., 1986), cigarette smoking combined with alcohol consumption (Stich and Rosin, 1983b), low levels of folic acid and vitamin B_{12} (Abe et al., 1984; Everson et al., 1988), and possibly gender (Norman et al., 1985), with women showing a slightly higher frequency of micronuclei than men. In addition, because the micronucleus assay is a nonspecific indicator of chromosome damage, exposures to chemicals, viruses, or x-rays other than the exposure of interest may increase the frequency of micronuclei.

Conclusion

The peripheral blood lymphocyte assay and exfoliated cell assay appear to be the most promising for future epidemiologic research. Each assay possesses a low level of invasiveness, is applicable to large human populations, and has been shown to indicate dose–response relationships. Although the exfoliated cell assay has been used in far more field applications than the lymphocyte assay, both need more field work to assess

the characteristics of each assay more fully. In particular, the variability of the assays and dose–response relationships could use further characterization. More work needs to be done with the exfoliated cell assay on cells other than buccal and urinary tract cells.

Questions That Can Be Addressed With the Micronucleus Assay

Micronuclei are indicators of chromosome damage due to genotoxic agents that cause chromosome breaks or spindle dysfunction. Micronuclei can serve as markers of exposure as well as markers of biological response, as the effectiveness of activation and deactivation mechanisms determine whether micronuclei form. Micronuclei may also be used to predict the carcinogenic potential of environmental agents, given that most carcinogens are found to be mutagens and that chromosomal aberrations are believed to be initial steps in carcinogenesis. It is not known, however, how the presence of micronuclei relates to the risk of cancer, although both micronucleus formation and the development of cancer are associated with x-rays, tobacco and alcohol use, and betel quid chewing. Thus, the question of the relationship between the frequency of micronuclei and the risk of cancer remains unanswered. Perhaps future epidemiologic studies can establish this link.

Epidemiologists seek to understand causes of disease. Hence, they try to determine whether associations exist between exposures and disease outcomes. Determining the utility of biological markers in epidemiologic research appears to be a three-step process. First, one needs to establish whether the exposure of interest leads to a detectable increase in the marker (frequency of micronuclei). Next, one needs to determine what relevance elevated levels of the marker have in future development of disease. Finally, one needs to characterize the dose–response relationship between exposure status, level of the marker, and risk of disease.

The most direct way of answering these questions is to periodically assess micronucleus frequencies in a group of people with known exposures to a genotoxic agent (one that is capable of elevating micronucleus frequencies) and determine whether the people with the highest micronucleus frequencies develop cancer. Unfortunately, this costly and time-consuming endeavor would require thousands of subjects, many years, and much personnel time.

Short-term projects are far more practical. Studies by Stich and Rosin (1982) have already shown that populations known to be at high risk of cancer and exposed to carcinogenic agents also have higher micronucleus frequencies in buccal mucosa cells. Future studies using elevated micronucleus frequencies as measures of outcome may identify tissues particularly susceptible to the carcinogenic effects of certain agents (Stich, 1986). Furthermore, as the test may be performed in a variety of cells and species, studies with micronuclei may serve as a link in risk assessment studies of cell cultures in vitro and in studies with animals and humans in vivo (Stich, 1986).

Because micronucleus assays in short-lived cells detect recent damage, the assays can be used as a measure of exposure that can be related to current disease status. Case-control studies of cancer patients, for example, might include assessments of current micronucleus frequencies. The relevance of such analyses depends on the nature of the association between current micronucleus frequencies and current cancer status.

Possibly of more interest would be case-control studies of cancer patients assessing

the relevance of past exposures. This could be accomplished with the use of cells stored for other purposes, assuming that a large number of cells were stored and that the people were exposed to some genotoxic agent. Given that lymphocytes are long-lived, perhaps using the micronucleus assay with peripheral blood lymphocytes can show the relevance of past exposures.

The micronucleus assay can also be used to ask questions associated with treatment efficacy. For example, Stich et al. (1984, 1985) and Muñoz et al. (1987) explored the utility of the micronucleus assay in assessing the efficacy of chemopreventive measures. These studies are particularly important because they show the effects of treatment in a relatively short period of time.

The micronucleus assay is a useful indicator of genotoxic damage from a number of environmental agents in a variety of cell types and populations. Improvements in the scoring of micronuclei through the standardization of criteria for their identification and through automation of the scoring process should enhance the usefulness of this promising assay. Applications of the micronucleus assay in population studies with different genotoxic exposures may augment our knowledge of the carcinogenic potential of environmental agents in humans, and may help elucidate mechanisms of carcinogenicity.

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