A simple, highly sensitive and improved method for the measurement of bleomycin-detectable iron: the ‘catalytic iron index’ and its value in the assessment of iron status in haemochromatosis

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ABSTRACT

In the presence of ferrous ions (Fe²⁺), the anti-tumour agent bleomycin will induce DNA degradation. Degradation of DNA into substances detectable by the thiobarbituric acid test has been used previously for the detection of iron in a form that is capable of catalysing the formation of the potentially harmful hydroxyl free radical. In the present paper, we describe the application of the ethidium-binding assay of DNA damage to the measurement of bleomycin-detectable iron, comparing its performance with the conventional method in the assessment of iron standard solutions and plasma samples from haemochromatosis patients. The ethidium-binding assay proved to be more responsive than the thiobarbituric acid test in the detection of DNA damage induced by very low concentrations of iron, but became saturated at higher iron concentrations. Agreement between the two versions of the assay in the identification of plasma samples containing bleomycin-detectable iron was good, but agreement on the actual concentrations of such iron in the positive samples was poor. This discrepancy is believed to be due to interference with the thiobarbituric acid assay by plasma. Consequently, it was not possible to obtain reliable estimates of free iron concentrations in plasma when using the conventional version of the bleomycin assay. We have devised a parameter of iron status called the catalytic iron index. For healthy, non-haemochromatotic individuals, the mean value of this parameter was found to be 0.81 (range 0.78–0.84; n = 20). Elevated values were observed in some plasma samples from haemochromatosis patients, but these showed no correlation with serum ferritin levels. In contrast, correlations were seen with both serum iron and transferrin saturation levels, but only when these were above the normal range.

INTRODUCTION

In 1981, an assay was described for the selective measurement of radical-promoting, loosely bound iron in biological fluids [1]. The assay, based on the ability of the anti-tumour agent bleomycin to degrade DNA in the presence of ferrous ions (Fe²⁺), is believed to indicate the presence in a biological sample of iron in a form that is capable of catalysing the generation of the extremely reactive and potentially harmful hydroxyl radical.

Key words: bleomycin assay, free radicals, haemochromatosis, hydroxyl radical, iron.
Abbreviations: BDI, bleomycin-detectable iron; CII, catalytic iron index; DTPA, diethylenetriaminepenta-acetic acid; MDA, malondialdehyde; TBA, thiobarbituric acid.
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‘OH) from superoxide and hydrogen peroxide [1–3]. Due to its sequestration in storage, transport and other iron-containing proteins, iron is believed to be essentially unavailable for the catalysis of ‘OH formation under most physiological conditions [4]. However, bleomycin-detectable iron (BDI) has been detected in several biological fluids, including sera from patients with idiopathic haemochromatosis [2] and thalassemia major [5], rheumatoid synovial fluid [1,2], normal human cerebrospinal fluid [6], plasma from both premature and full-term infants [7], and in patients undergoing cardiopulmonary bypass surgery [8] or chemotherapy [9,10]. An increase in BDI has also been detected in rat kidneys following ischaemia/reperfusion [11].

Several lines of evidence indicate that ‘OH formation may be responsible for the harmful effects of excessive iron [4,12–14]. Therefore the determination of BDI may be of particular clinical relevance, in that it is a functional assay for the ability of a biological sample to support ‘OH formation: the methods currently employed in clinical medicine for the determination of iron status, including serum iron, ferritin and transferrin saturation levels, reflect primarily total body iron stores and fluxes, and therefore provide only an indirect indication of the potential for ‘OH formation and consequent oxidative damage to biological molecules [15–18]. Indeed, it has been reported that, of a series of biochemical markers measured in rats following dietary iron loading, only serum iron levels were raised above the values determined for control-fed animals [13]. Despite the detection of ‘OH formation in vivo in the iron-fed rats, but not in the control animals, no significant decrease in total iron-binding capacity was seen [13]. In a similar study, despite convincing evidence for free-radical formation and biomolecular damage in vivo, no increases in serum iron or total iron-binding capacity were detected in the iron-loaded animals [14].

In the conventional bleomycin assay, DNA is incubated with ascorbic acid (to reduce Fe⁺ ions), bleomycin and the biological sample. DNA damage is then measured as the formation of malondialdehyde (MDA) from the 2′-deoxyribose moiety of the DNA using the thiobarbituric acid (TBA) test [1–3]. This involves the formation of a chromophore, the (TBA)_2–MDA adduct, which is measured at 532 nm. The concentration of BDI present in the original sample is then obtained from this reading using a standard curve. Formation of the (TBA)_2–MDA adduct requires relatively harsh conditions, and it is widely recognized that complex secondary reactions occur during the heating stage of the assay, which may result in the generation of misleading data. Studies with peroxidizing lipids, for example, have shown that much of the MDA incorporated into the (TBA)_2–MDA adduct is formed from lipid hydroperoxides during the heating stage of the assay [19,20]. These secondary reactions are affected markedly by metal ions (Fe and Cu), chelating agents, hydrogen peroxide and antioxidants [19,20]. Therefore there exists the possibility that the findings from studies in which the TBA test is used to investigate the effects of such reagents on MDA formation (from lipids and DNA) may, in fact, relate as much to their effects on the heating stage of the TBA test as to the process under investigation. When using the bleomycin assay, for example, it is important to establish that the MDA detected originates from the DNA and not from lipid hydroperoxides formed during iron-catalysed lipid peroxidation. Indeed, elevated (approx. 27-fold) levels of lipid hydroperoxides have been reported in the plasma of rats with dietary-induced iron overload [14]. Furthermore, in human subjects with iron overload, plasma TBA reactivity (MDA is not the only product of lipid peroxidation detected by the TBA test) is associated with the presence of BDI [21].

To avoid potential problems associated with the TBA test, we have devised a modified version of the assay in which DNA degradation resulting from the interactions between bleomycin, DNA, ascorbic acid and iron is measured using the ethidium-binding assay for DNA damage. The ethidium-binding assay is based on the principle that damage to DNA results in a compromise in its ability to enhance the fluorescence of the intercalating dye ethidium bromide. The assay was developed originally for the study of DNA damage by radicals generated using γ-radiolysis [22], but has found widespread application to metal-dependent systems of radical formation [23–25]. The assay is very simple and requires none of the harsh conditions employed in the TBA test. In the present paper, we compare the performance of the new assay with that of the conventional bleomycin assay in the measurement of BDI in standard solutions and in plasma from patients with hereditary haemochromatosis.

**MATERIALS AND METHODS**

**Reagents and preparation of stock solutions**

All solutions were prepared using Millipore-filtered deionized water. DNA (sodium salt, from either salmon testes or calf thymus; Sigma Chemical Co.) was prepared as a 1 mg/ml stock solution. Chelating resin (Sigma) was then added to remove contaminating metal ions [26]. After leaving overnight at 4 °C followed by low-speed centrifugation, aliquots of the DNA solution were pipetted directly from above the chelating resin into disposable plastic tubes in which the reactions were to be performed. Bleomycin sulphate (Sigma) was dissolved in water that had been treated with chelating resin (as described for the DNA solution) to give a 1 unit/ml stock solution. This was divided into aliquots and stored at −80 °C in microcentrifuge tubes. Tris/HCl (from Boehringer Mannheim) was prepared as both 0.4 M stock and 1.0 M stock solutions, pH 7.4, and stored at 4 °C.
Measurement of DNA damage by iron–bleomycin using the ethidium-binding assay

Incubations were prepared by the addition, in the order given, of reagents from the stock solutions described above to disposable plastic tubes: 100 μl of DNA, 950 μl of water, 100 μl of bleomycin, 500 μl of 0.4 M Tris/HCl, 200 μl of MgCl₂·6H₂O, 100 μl of iron standard and 50 μl of 40 mM ascorbic acid. These ‘reacting’ tubes were then incubated at 37 °C for 2 h, followed by the addition of 100 μl of DTPA.

Since the ability of DNA to enhance the fluorescence of ethidium bromide is a measure of its integrity, separate ‘0%’ and ‘100%’ fluorescence control incubations were carried out for each concentration of iron used. The 0% fluorescence tubes were prepared as described above for the iron-standard reaction mixtures, except that the DNA was replaced with water (1050 μl total) and DTPA was added after the water, but before the bleomycin and other reagents. The tubes were then incubated as above, but no DTPA was added after the incubation. The 100% fluorescence tubes were prepared as for the iron-standard reaction mixtures, but the order of reagent addition was changed: DTPA was added after the DNA and water, but before the bleomycin and other reagents. Again the tubes were incubated as above, but DTPA was not added after the incubation. Following incubation, 5 μl of ethidium bromide was added to all tubes. Fluorescence readings were then taken (excitation 510 nm; emission 590 nm). Subtraction of a given 0% reading from the corresponding (i.e. same concentration of iron) 100% reading gives the value for 100% fluorescence enhancement of ethidium bromide by the (undamaged) nucleic acid. Similarly, subtraction of a given 0% reading from the corresponding reacting tube is a measure of the ability of the DNA in the reacting tube to enhance the fluorescence of the dye, which is then expressed as a percentage of the value obtained from the 100% incubation.

Use of the bleomycin assay based on the TBA test

DNA damage by bleomycin/iron and BDI in human plasma were also measured using the conventional bleomycin assay, as described elsewhere [2,3,10]. Slight variations exist in the methodology reported in the literature. To avoid a potential source of contaminating iron, Evans and Halliwell [3] recommended that the assay be performed without the use of a buffer. However, without a buffer, we experienced considerable difficulty in the achievement of a stable pH. Although results similar to those reported herein were also obtained using a buffer-free system, we preferred to use a buffer. Gutteridge and Halliwell [2] have reported that Tris is a suitable buffer for the bleomycin assay, having no effect on DNA damage by iron–bleomycin and TBA reactivity. Indeed, others have used this buffer in the assay without comment [10]. We prepared a 1 M stock solution of Tris/HCl, pH 7.4, which was treated with chelating resin...
before use. The fact that the background levels of DNA damage we obtained (see Figure 1B) are well within those reported by Evans and Halliwell [3] indicates that our use of the buffer did not cause significant iron contamination. The final reagent concentrations used were: 100 mM Tris/HCl, pH 7.4, 0.5 mg/ml DNA, 5 mM MgCl₂ and 0.795 mM ascorbic acid, as described by Evans and Halliwell [3] (except the buffer).

Other measurements of iron status
The other parameters of iron status reported in Table 1 and Figure 2 were determined in the clinical biochemistry laboratories at Aberdeen Royal Infirmary and Hairmyres Hospital, as considered necessary for clinical purposes.

RESULTS
Detection of DNA damage induced by bleomycin in the presence of iron standards
Incubation of DNA with bleomycin, ascorbic acid and increasing amounts of iron resulted in damage to the nucleic acid, indicated as a compromise in its ability to enhance the fluorescence of ethidium bromide (Figure 1A). Despite attempts to remove contaminating iron from the reagents using chelating resin, DNA incubated in the absence of added iron showed an approximate 14% decrease in its ability to enhance the fluorescence of the dye, indicating the presence of traces of contaminating iron. The assay was particularly responsive to iron at concentrations up to approx. 50 nM, above which it became progressively more saturated (Figure 1A). The sensitivity of the assay to iron at higher concentrations could be improved simply by increasing the concentration of DNA used, but this resulted in poorer sensitivity to iron at lower concentrations (results not shown).

For comparison, a standard curve was also prepared using the conventional bleomycin assay, based on the TBA test, using identical concentrations of iron (Figure 1B). Although slightly less sensitive to iron at the very low concentrations, the response of the assay was linear over the full concentration range. The small absorbance at 532 nm observed from incubations performed in the absence of added iron indicates the presence of traces of contaminating iron in the reagents, as seen with the ethidium-binding assay.

BDI in plasma from haemochromatosis patients
The abilities of the ethidium-binding assay and the TBA method to measure BDI in human plasma were compared using samples from a group of ten haemochromatosis patients. Haemochromatosis patients have been employed previously in the investigation of BDI [2]. The patients selected for study displayed a broad range of serum ferritin concentrations, from those having values below the normal physiological range (20–350 ng/ml) to those with values well in excess of this. Similarly, the patients displayed transferrin saturation values spanning and exceeding the physiological range of 20–50%. As shown in Table 1, good agreement was found between the two assays in the determination of which plasma samples contained BDI, which were found to be those having both serum ferritin and transferrin saturation values above the respective normal ranges. Plasma from patient 3, which displayed an elevated serum ferritin level but abnormally low transferrin saturation, contained no BDI. Agreement between the two assays on the actual concentrations of iron present in the positive samples was, however, poor: in some samples, the ethidium-binding assay reported the higher concentration, whereas in other samples the higher concentration was reported by the TBA assay. Both assays reported BDI concentrations of ‘less than zero’ in five of the samples, meaning that they protected DNA from damage induced by the traces of contaminating iron present in the reagents (i.e. the levels of DNA damage measured were less than that induced by ‘0 nM added iron’, the reagent blank; see below).
Table 1  Comparison of the ethidium-binding and TBA-based assays in the measurement of BDI in plasma from haemochromatosis patients

In both assays, 50 µl of plasma was used per ml of incubation, except for samples from patients 5, 8, 9 and 10, for which 10 µl of plasma was used per ml of incubation in the ethidium-binding assay. Concentrations reported as < 0 indicate that the fluorescence or absorbance value was respectively above or below that obtained from the reagent-blank incubation. Values are means ± S.D. (n = 3). n.d., not determined.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serum ferritin (ng/ml)</th>
<th>Transferrin saturation (%)</th>
<th>Ethidium-binding method</th>
<th>TBA-based method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>31</td>
<td>&lt; 0</td>
<td>&lt; 0</td>
</tr>
<tr>
<td>2</td>
<td>&gt; 1000</td>
<td>99</td>
<td>0.4 ± 0.0</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>575</td>
<td>8</td>
<td>&lt; 0</td>
<td>&lt; 0</td>
</tr>
<tr>
<td>4</td>
<td>n.d.</td>
<td>6</td>
<td>&lt; 0</td>
<td>&lt; 0</td>
</tr>
<tr>
<td>5</td>
<td>830</td>
<td>98</td>
<td>1.7 ± 0.2</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>19</td>
<td>&lt; 0</td>
<td>&lt; 0</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>11</td>
<td>&lt; 0</td>
<td>&lt; 0</td>
</tr>
<tr>
<td>8</td>
<td>1162</td>
<td>94</td>
<td>1.7 ± 0.1</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>9</td>
<td>752</td>
<td>99</td>
<td>3.9 ± 0.1</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>884</td>
<td>96</td>
<td>1.6 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Normal range</td>
<td>20–350</td>
<td>20–50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2  Effects of plasma dilution factor on the measurement of BDI using the TBA and ethidium-binding versions of the bleomycin assay

Plasma from a haemochromatosis patient (patient 10 in Table 1) was assayed at the dilutions indicated. Values are means ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Plasma dilution (µl/ml of incubation)</th>
<th>TBA-based method</th>
<th>Ethidium-binding method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA damage (A532)</td>
<td>Plasma BDI (µM)</td>
</tr>
<tr>
<td>10</td>
<td>0.065 ± 0.015</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>25</td>
<td>0.218 ± 0.056</td>
<td>4.5 ± 1.2</td>
</tr>
<tr>
<td>50</td>
<td>0.167 ± 0.020</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>100</td>
<td>0.034 ± 0.045</td>
<td>0.1 ± 0.0</td>
</tr>
</tbody>
</table>

The values reported in Table 1 for the TBA-based bleomycin assay were obtained using 50 µl aliquots of plasma, diluted to 1 ml, as in the published method [3]. However, when the volume of plasma used from one of the BDI-positive patients (patient 10) was halved, the level of DNA damage detected was found to increase, resulting in a much higher value for the plasma BDI concentration (after correction for dilution). Similarly, doubling the volume of plasma assayed resulted in a decrease in the levels of DNA damage and iron detected (Table 2). In contrast, increasing the volume of plasma assayed from the same patient from 10 µl to 25 µl per ml of incubation, but using the ethidium-binding method, resulted in an increase in the level of DNA damage recorded. After correction for dilution, the calculated plasma BDI concentration remained unchanged, at ~ 1.6 µM (Table 2). Increasing further the volume of plasma used in the ethidium-binding assay caused no further increase in DNA damage (Table 2), reflecting saturation of the assay (see above and Figure 1A). Thus the BDI concentrations calculated from the fluorescence enhancement values obtained using 50 or 100 µl of plasma per ml of incubation (Table 2) are inappropriately low, simply because progressively lower dilution factors were used to correct iron concentrations calculated from the same fluorescence enhancement value (~ 28%). This reasserts the importance of the precautionary measure mentioned above (see the Materials and methods section): any plasma sample yielding a fluorescence enhancement value of ~ 30% or less should be assayed again at greater dilution.

The discrepancies encountered when assaying different volumes of plasma in the original version of the bleomycin assay, which are suggested to reflect the effects of plasma components on the heating stage of the TBA test (see Discussion), mean that the measured absorbance
values at 532 nm cannot be reliably converted into iron concentrations using a conventional standard curve. Attempts to prepare an appropriate standard curve using iron standards in the presence of plasma would be flawed due to iron sequestration in transferrin, which would vary from patient to patient.

The catalytic iron index (CII)

The BDI concentrations reported in Table 1, obtained using the ethidium-binding assay, were calculated from a standard curve after correction for contaminating iron in the reagent-blank incubation (‘0 nM iron’). This approach, however, is not entirely satisfactory because, as mentioned above, it resulted in the reporting of BDI concentrations of ‘less than zero’ in half of the samples. Fluorescence (%) values that are higher than that obtained from the reagent-blank incubation (~ 86% in Figure 1A), reporting the presence in plasma of BDI at concentrations of ‘less than zero’, result from protection of the nucleic acid from damage induced by background iron. This protection is believed to be due to sequestration of the contaminating iron by transferrin. Indeed, all of the samples for which the BDI was ‘less than zero’ had transferrin saturation levels within or below the normal, physiological range (20–50%), indicating spare iron-binding capacity.

The fluorescence value obtained following the incubation of DNA, bleomycin and a given plasma sample will be determined by at least three major factors: (i) the concentration of free iron in the plasma; (ii) the concentration of contaminating iron from the reagents; and (iii) the ability of the plasma to sequester free iron in proteins such as transferrin. The last of these is of clear relevance; indeed, total iron-binding capacity and transferrin saturation are recognized as important clinical parameters of iron status. Since reagent-contaminating iron levels are expected to vary between assays and laboratories, it was considered appropriate to introduce a parameter for plasma ‘catalytic’ iron status that is corrected for contaminating iron levels and reflects both (i) and (iii) above. Therefore we have devised a parameter called the catalytic iron index (CII). The CII is calculated by division of the fluorescence reading obtained from the reagent-blank incubation by the reading obtained from the incubation containing plasma. For example, DNA incubated with bleomycin and plasma from patient 2 (Table 1) retained 37.3% of its ability to enhance the fluorescence of ethidium bromide, whereas DNA incubated without plasma (the reagent-blank) retained 86.2% of this ability, giving a CII value of 2.3. The subscripted 50, the dilution factor, indicates that 50 μl of plasma was used per ml of incubation; only CII values with the same dilution factor can be compared.

Clinical evaluation of the CII

In order to evaluate in greater detail the possible clinical value of the CII, measurements were made on 25 plasma samples collected over a 2-year period from four haemochromatosis patients. During this period, the patients received treatment by venesection, involving the removal of 350–500 ml of blood, at a frequency of only a few occasions a year up to weekly, depending solely on conventional estimates of iron status. For comparison, samples were also collected from 20 healthy, non-haemochromatotic volunteers. In this study, 450 μl of plasma was used per ml of incubation. The average CII value determined for the 20 non-haemochromatotic individuals was 0.81 (range 0.78–0.84). The fact that this
value is below unity reflects the capacity of plasma to counteract the undesirable effects of trace levels of iron introduced by the reagents. In contrast, although some of the samples from haemochromatosis patients also gave CII values close to 0.81, elevated values were common. The CII values from the patients are presented in Figure 2, where they are plotted separately against three conventional measures of iron status: serum ferritin, serum iron and transferrin saturation. Although serum ferritin values were not available for all 25 samples, it can be seen from the samples that are shown that this measurement bears no correlation with the CII values (Figure 2A). In contrast, those serum iron and serum transferrin values that were above the normal range did show correlations with CII, particularly the former (Figures 2B and 2C respectively). It is noteworthy that none of the samples that had serum iron or transferrin saturation levels within the normal range displayed a CII value greater than 1, indicating a capacity to sequester contaminating iron.

DISCUSSION

Our findings demonstrate that the ethidium-binding assay can be used to detect DNA damage induced by iron in the presence of bleomycin and ascorbic acid. The assay was found to be particularly responsive to damage induced by iron at very low concentrations (up to approx. 50 nM), but saturation occurred at higher concentrations. In contrast, although somewhat less sensitive to iron at very low concentrations, DNA damage assessed using the TBA method proved to be linear, even at the highest concentrations tested. This difference in the sensitivity ranges of the two assays is believed to reflect the concentration of DNA used in each method. In the TBA-based bleomycin assay, the final concentration of DNA in the incubated reaction is 0.5 mg/ml, whereas a concentration of only 0.05 mg/ml was used in the ethidium-binding version of the assay described here. Thus, in the latter method, it appears that DNA becomes limiting at an iron concentration between 50 and 100 nM. In contrast, ten times as much DNA is employed in the TBA-based method. Although the concentration of DNA employed in the ethidium-binding version of the assay can be increased, this results in decreased sensitivity to low concentrations of iron. This is because the degree of DNA damage induced by a given amount of iron, expressed as percentage total fluorescence enhancement, will be lower if the total amount of DNA present is increased. This problem does not arise with the TBA test, because it is based on the measurement of product accumulation rather than the percentage of DNA remaining intact. Thus each version of the bleomycin assay, when applied to iron standards, has an advantage and a shortcoming compared with the other. A particular advantage of the ethidium-based assay is its simplicity: whereas the conventional assay involves acidification and heating at 80 °C for 15 min with TBA followed by extraction into butanol, no post-incubation treatment (other than the addition of 5 μl of ethidium bromide solution) is required in the revised assay.

The ethidium-binding assay also proved to be of value in the measurement of BDI in human plasma. Although the method gave good agreement with the conventional bleomycin assay in the detection of samples positive for BDI, agreement on the actual concentrations of BDI reported by the two assays was not always satisfactory. That the discrepancies between the two methods arose from a problem associated with the TBA test, and not the ethidium-binding assay, became apparent when various volumes of plasma were assayed: increasing the volume of plasma included in the 1 ml incubation from 10 to 25 μl resulted in an increase in the level of DNA damage observed, but increasing the volume of plasma further resulted in a decrease in damage. Thus the BDI concentration of 0.9 μM obtained for a particular patient using 50 μl of plasma in the conventional bleomycin assay (Table 1) is somewhat arbitrary, in that higher values were obtained when using lower volumes of plasma (Table 2).

As described in the Introduction, the reactions that lead to formation of the (TBA)₂–MDA adduct during the heating stage of the TBA test are complex and subject to interference from antioxidants and metal chelators [19,20]. Following the incubation of DNA with bleomycin in the presence of plasma containing BDI, it is expected that only a small proportion of the total MDA eventually detected as the TBA adduct will be present initially: most of the MDA detected will be generated during the subsequent heating stage of the assay, during which hydroperoxide derivatives of the nucleic acid (centred on the 2'-deoxyribose moiety) will undergo further degradation to MDA and other aldehydic products. These degradation reactions involve free radicals and are catalysed by metal ions. When using the TBA test to measure lipid peroxidation, it is common to add the antioxidant butylated hydroxytoluene to suppress these chain reactions and thereby avoid the overestimation of MDA [27,28]. In the bleomycin assay, however, these reactions are probably necessary for optimal sensitivity. We propose that antioxidants present in plasma (including protein thiols) are responsible for the progressive loss of sensitivity of the TBA test seen with increased sample volumes. This is probably not the only factor responsible for the variation in sensitivity of the conventional bleomycin assay with plasma volume, and it is expected that the volume of plasma that is optimal for formation of the (TBA)₂–MDA adduct will vary from patient to patient, depending on the actual level of BDI present, antioxidant status and transferrin saturation. Because of such considerations, it is very difficult to
envisage how levels of \((TBA)_2\text{-MDA}\), generated from DNA in the presence of plasma can be related to BDI concentrations using a standard curve. Plasma cannot be included in the standard curve incubations, because the iron would be sequestered by transferrin, the extent of which would depend on the level of transferrin saturation. In contrast, providing that saturating levels of DNA damage are avoided, the ethidium-binding version of the bleomycin assay is free from any such problems. It is essential, however, that any sample giving a fluorescence enhancement value of 30% or below should be diluted and measured again.

Although TBA-reactive substances generated from lipid hydroperoxides could, in principle, lead to misleadingly high BDI values in the conventional bleomycin assay, all of the plasma samples tested here gave very low absorbance readings at 532 nm after processing through the TBA test (M. J. Burkitt, unpublished work). In any case, the conventional bleomycin assay gave BDI concentrations above those reported by the ethidium-based assay for only two of the samples tested. Therefore, although the possibility of a contribution from lipid-hydroperoxide-derived TBA-reactive substances should always be considered when performing the conventional bleomycin assay (see, e.g., Peters et al. [21]), this appeared not to be an important factor in the samples tested here.

Using both versions of the bleomycin assay, plasma from healthy, non-haemochromatotic individuals failed to induce damage to DNA, indicating the absence of BDI. Moreover, such plasma was found to protect DNA from background levels of damage caused by contaminating iron. Although plasma contains radical-scavenging antioxidants, DNA damage by iron–bleomycin is known to occur by a site-specific mechanism, against which such antioxidants fail to offer protection [2]. Indeed, antioxidants that have reducing properties (e.g. phenolics) are reported to enhance DNA damage by iron–bleomycin [29]. Hence we conclude that the protective effect of normal plasma reflects its ability to sequester traces of contaminating iron in a form that is unavailable for binding to bleomycin, probably in transferrin, which is saturated only partially in healthy individuals.

In order to take into account the ability of plasma from healthy individuals to inactivate adventitious iron introduced from the reagents (which, realistically, cannot be avoided), we have introduced a parameter called the CII. Elevated CII values were often observed in plasma taken from haemochromatosis patients, and indeed were found to show correlations with serum iron and transferrin saturation values, but only when these values were above the normal, physiological range. The management of the patients included in the present study aimed broadly at weekly venesection to bring about mild anaemia, following which the frequency of venesection was reduced to between four and eight times per year. There are clinicians who advocate that the aim of venesection should be to maintain low serum ferritin levels (less than 50–100 ng/ml) [30]. Others maintain that serum ferritin is not a sensitive index of iron stores in the context of the haemochromatosis patient who has not been fully ‘de-ironed’, and that transferrin saturation offers a more accurate guide to the requirement for further venesection [17]. Although the results of the CII determinations in the present study were not used to modify the intensity of venesection, retrospective examination of the data provides support for the superiority of transferrin saturation (and, indeed, serum iron) values compared with ferritin levels in determining the frequency of venesection.

In addition to the assessment of iron status in haemochromatosis patients, the CII may also prove to be of value in connection with other pathological conditions. There is controversy in the literature regarding the relationship between iron and certain conditions that may involve excessive free-radical formation, including diabetes [31,32], atherosclerosis, coronary heart disease and myocardial infarction [33–38]. We propose that one of the reasons for this controversy is that the various conventional indices of iron status used in these studies (serum transferrin, transferrin saturation, serum iron, total iron-binding capacity and ferritin) give only indirect indications of the potential for iron-dependent free-radical formation and biomolecular damage in vivo. We suggest that application of the improved bleomycin assay described here may provide a more reliable indication of the relationship between iron and free-radical disease.

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