cysts from human feces rapidly (>90% in 7 hours) lose the ability to excyst at 37°C and require 2 to 7 days at low temperature (4° to 8°C) to excyst efficiently. We have not yet determined whether cysts induced in vitro are capable of excystation and are infective for animals. However, the ability to induce encystation in vitro will make it possible to obtain cysts without fecal contamination for studies of their basic biochemistry, of improved methods for disinfection of water, and of possible means of interrupting the life cycle of this parasite.

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25. Supported by Environmental Protection Agency CR 811950-01-1 and NIH grants AI 19863 and AM 35108. We thank A. Zenian for antibodies to *G. lamblia* trophozoites, E. Cabib for advice on the chitin synthetase assay and purified chitinase, Bayer Inc. for Nikkomycin, and A. Hofmann, E. Ziegler, P. Hagblom, C. Davis for comments, and S. McFarlin for typing the manuscript.

2 June 1986; accepted 11 December 1986

Bilirubin Is an Antioxidant of Possible Physiological Importance

ROLAND STOCKER, YORIHIRO YAMAMOTO, ANTONY F. MCDONAGH, ALEXANDER N. GLAZER, BRUCE N. AMES

Bilirubin, the end product of heme catabolism in mammals, is generally regarded as a potentially cytotoxic, lipid-soluble waste product that needs to be excreted. However, it is shown here that bilirubin, at micromolar concentrations in vitro, efficiently scavenges peroxyl radicals generated chemically in either homogeneous solution or multilamellar liposomes. The antioxidant activity of bilirubin increases as the experimental concentration of oxygen is decreased from 20% (that of normal air) to 2% (physiologically relevant concentration). Furthermore, under 2% oxygen, in liposomes, bilirubin suppresses the oxidation more than α-tocopherol, which is regarded as the best antioxidant of lipid peroxidation. The data support the idea of a "beneficial" role for bilirubin as a physiological, chain-breaking antioxidant.

ALTHOUGH MOST OF THE MOLECULAR oxygen in aerobic eukaryotic cells and organisms is sequentially reduced to water via the respiratory chain, both the univalent and bivalent reduction of oxygen occur during normal intermediary metabolism to give rise to superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (*1*). These oxygen reduction products have the potential to generate other reactive oxygen species (ROS) such as the hydroxyl radical, which then may initiate a radical chain reaction leading to extensive formation of lipid hydroperoxides. Lipid peroxides and their breakdown products alter the physicochemical properties of biomembranes and can cause damage to membrane-bound enzymes as well as to other macromolecules (*2, 3*). ROS have been implicated in the cause or pathology of cancer, aging, tumor promotion, heart disease, chronic inflammation, and parasitic infections (*2–4*). To prevent the formation of oxidants as well as to repair oxidative damage to tissues and macromolecules, all aerobic living organisms possess a complex armory of enzymatic and nonenzymatic antioxidant defenses. This includes the enzymes superoxide dismutase and catalase, the glutathione cycle, vitamins E and C, and β-carotene (*1–9*).

Work from this laboratory (*10*) has suggested that uric acid, the end product of purine metabolism, which is a powerful antioxidant (*10, 11*), may serve as a protective agent in human plasma. Recently, the antioxidant and membrane-protective properties of taurine, the end product of oxidative metabolism of cysteine, were reviewed (*12*). Another major metabolic pathway in mammalian systems is the degradation of protoheme derived from hemoproteins such as hemoglobin and cytochrome P-450 to bilirubin. As a result of this, approximately 300 mg of bilirubin per day are produced by normal adult humans (*13*). Bilirubin is generally regarded as a toxic compound when accumulated at abnormally high concentrations in biological tissues and is responsible for the clinical symptoms of kernicterus (*13, 14*). However, it has also been suggested that the bile pigments bilirubin and biliverdin may protect vitamin A and linoleic acid from oxidative destruction in the intestinal tract (*15*). Indeed, bilirubin contains an extended system of conjugated double bonds and a reactive hydrogen atom and thus could possess antioxidant properties. Therefore, we examined the antioxidant activity of bilirubin in an in vitro system where lipid was oxidized by a free radical chain mechanism.

The free radical chain oxidation of linoleic acid (LH) gives linoleic acid hydroperoxide (LOOH) quantitatively at the initial stage (*16*). This enabled us to follow the oxidation of linoleic acid simply by measuring the formation of LOOH. To get a constant rate of initiation (reaction 1) and, subsequently, of oxidation (reactions 2 and 3), we used the radical initiator 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) (*16*).

$$\text{AMVN} \xrightarrow{\Delta, O_2} \xrightarrow{\text{LH}} \text{L}\cdot \quad (1)$$

$$\text{L}\cdot + O_2 \rightarrow \text{LOO}\cdot \quad (2)$$

$$\text{LOO}\cdot + \text{LH} \rightarrow \text{LOOH} + \text{L}\cdot \quad (3)$$

The effects of bilirubin and its metabolic precursor biliverdin on the rate of peroxyl

R. Stocker, Y. Yamamoto, B. N. Ames, Department of Biochemistry, University of California, Berkeley, CA 94720.
A. F. McDonagh, Department of Medicine and The Liver Center, University of California, San Francisco, CA 94143.
A. N. Glazer, Department of Microbiology and Immunology, University of California, Berkeley, CA 94720.

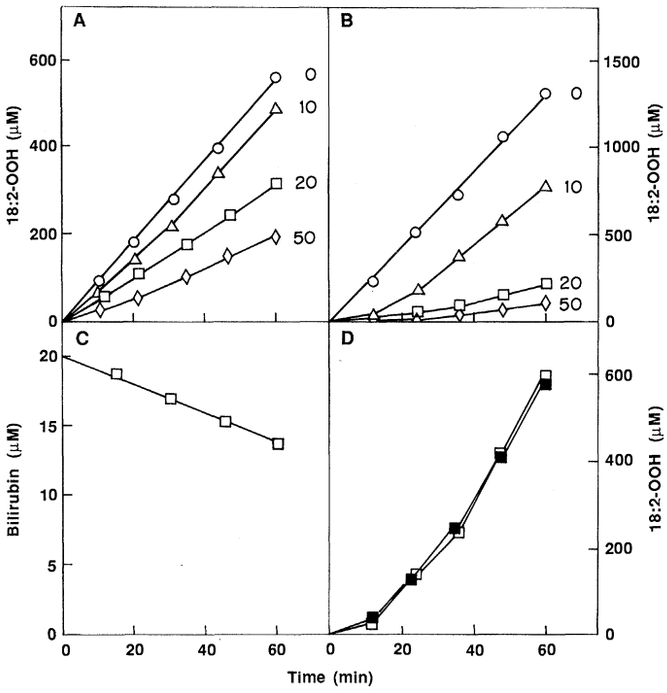

Fig. 1. AMVN-initiated oxidation of purified linoleic acid (*29*) in the presence and absence of heme degradation products under air at 37°C. The formation of linoleic acid hydroperoxide (18:2-OOH) was analyzed by high-pressure liquid chromatography (HPLC) equipped with an LC-18 column (Supelco, Bellefonte, PA). Methanol (1 ml/min) was used as the eluant and monitoring was at 234 nm (*29*). Under these conditions, 18:2-OOH eluted close to the solvent front. However, the contribution of the latter to the overall absorbance of 18:2-OOH at 234 nm was small and eliminated by subtraction. AMVN (Polysciences, Warrington, PA) was recrystallized from hot methanol. Effect of (**A**) recrystallized bilirubin (*30*) and (**B**) biliverdin IX dihydrochloride (Porphyrin Products, Logan, UT) at 0 μ*M* (○), 10 μ*M* (△), 20 μ*M* (□), and 50 μ*M* (◇) on the AMVN- (1.25 m*M*) initiated oxidation of linoleic acid (158 m*M*). The reaction solvent was chloroform in (A) and methanol in (B). (**C**) AMVN- (1.25 m*M*) induced disappearance of bilirubin (20 μ*M*) (□) in the presence of linoleic acid (158 m*M*) under the conditions described for (A). Bilirubin was quantitated at 460 nm by HPLC (LC-18 column) with 0.1*M* di-*n*-octylamine acetate in methanol (*p*H 7.7) as the eluant (*19*). (**D**) Effect of bilirubin (□) and its 4*Z*,15*E* and 4*E*,15*Z* configurational photoisomers (■) (*19*) on the AMVN- (1.25 m*M*) initiated oxidation of linoleic acid (158 m*M*) in chloroform and methanol (1/1, v/v). Recrystallized bilirubin was dissolved in chloroform and triethylamine (1/1, v/v) and portions were removed before and after exposure to blue light for 10 minutes (*19*). The solvents were dried under a stream of nitrogen (*31*) before the reaction solution was added. The final concentration of bilirubin was 20 μ*M*. The data shown represent typical results obtained for each of the experiments, with a variance of less than 5% (n = 3 or 4).

radical–induced oxidation of linoleic acid in homogeneous solution are summarized in Fig. 1. In the absence of heme metabolites the oxidation proceeded smoothly and at a constant rate. Both bilirubin and biliverdin at micromolar concentrations inhibited the formation of LOOH significantly and in a concentration-dependent way (Fig. 1, A and B). When one compares the relative antioxidant activity of bilirubin in chloroform (Fig. 1A) with that of biliverdin in methanol (Fig. 1B) it appears that the latter is a more efficient peroxyl radical trap than the former (*17*). Under identical conditions, 20 μ*M* bilirubin decreased the formation of LOOH by about 200 μ*M* within the first 60 minutes (Fig. 1A) while within the same time only 6 μ*M* bilirubin was consumed (Fig. 1C). This suggests that under these conditions bilirubin competed with linoleic acid for the linoleic acid peroxyl radical (LOO·) in reaction 3, resulting in partial inhibition of the chain reaction.

These data indicate that bilirubin at micromolar concentrations can scavenge the chain-carrying peroxyl radical either by donating a hydrogen atom attached to the C-10 bridge of the tetrapyrrole molecule to form a carbon-centered radical (BR·) with resonance stabilization extending over the entire bilirubin molecule (reaction 4) or by some other path (*2*).

$$\text{LOO}\cdot + \text{BR} \rightarrow \text{LOOH} + \text{BR}\cdot \qquad (4)$$

The former mechanism is supported indirectly by the finding that concomitant with the decrease in absorbance at 450 nm, as a result of bilirubin oxidation, a new peak appeared at wavelengths >550 nm. BR· may then react with either another peroxyl radical to give rise to a nonradical product (reaction 5), or oxygen (reaction 6):

$$\text{BR}\cdot + \text{LOO}\cdot \rightarrow \text{BR–OOL} \qquad (5)$$

$$\text{BR}\cdot + \text{O}_2 \rightleftarrows \text{BR–OO}\cdot \qquad (6)$$

By analogy to model compounds with conjugated polyunsaturation (*9, 18*), the antioxidant activity of biliverdin (BV) is probably due to the resonance-stabilized, carbon-centered radical (or radicals) formed by the addition of an LOO· to the pigment (reaction 7):

$$\text{LOO}\cdot + \text{BV} \rightarrow \text{LOO–BV}\cdot \qquad (7)$$

The relatively higher radical-trapping activity of biliverdin compared to bilirubin (Fig. 1, A and B) suggests that the rate constant for the addition of LOO· to biliverdin (reaction 7) is faster than the rate constant of reaction 4.

Bilirubin photochemistry has attracted wide attention in the context of the treatment of jaundiced neonates by prolonged irradiation with visible light (*19, 20*). Such irradiation results in configurational and structural isomerization of the bilirubin molecule with disruption of the usual hydrogen-bonded system and formation of isomers sufficiently polar to be excreted readily (*19, 20*). Phototherapy thereby reduces the concentration of circulating bilirubin and the concomitant risk of encephalopathy associated with high concentrations of the pigment, particularly in the brain (*20*). Recently, a photoisomer of bilirubin was detected in the plasma of healthy adults after exposure to sunlight (*21*), indicating that photoreactions of bilirubin may be of more general importance. Therefore, we examined whether the observed inhibitory effect of bilirubin on linoleic acid oxidation was altered upon irradiation of the pigment with blue light to a photoequilibrium mixture of configurational isomers. Clearly, this was not the case, as shown in Fig. 1D, indicating that photoisomerization of bilirubin did not affect its antioxidant properties in homogeneous solution.

To assess the relative importance of bilirubin as a possible physiological antioxidant, we compared its peroxyl radical trapping activity with that of β-carotene and α-tocopherol (Fig. 2). In homogeneous solution and under oxygen concentration of air, bilirubin at 10 μ*M* reduced the rate of oxidation of linoleic acid by about 16%, compared to 10% by 10 μ*M* β-carotene (Fig. 2A). α-Tocopherol at the same concentration was much more efficient than β-carotene and bilirubin, inhibiting the initial rate by 97% (Fig. 2A). It has been shown previously (*22*) that in homogeneous solution α-tocopherol reacts with the chain-carrying radical so efficiently that the chain of oxidation is interrupted almost completely, thereby producing a clear induction period. Once α-tocopherol is consumed (the breakpoint in Fig. 2A), the rate of oxidation is the same as that observed in the absence of any antioxidant.

When used at the same concentration but under 2% oxygen, comparable to what is actually found in tissues, the antioxidant

activity of bilirubin increased, as shown by the 35% inhibition of the rate of oxidation of linoleic acid (Fig. 2B). This may be explained by an equilibrium of reaction 6 favoring the bilirubin radical under low oxygen concentration. Such an oxygen-dependent alteration in peroxyl radical trapping activity was also observed with β-carotene (Fig. 2B), as has been reported previously (*9*). In contrast to bilirubin and β-carotene, the inhibition of the initial rate of oxidation of linoleic acid in homogeneous solution by α-tocopherol was similar under the different oxygen concentrations (Fig. 2, A and B).

The oxidations of polyunsaturated fatty acids and phosphatidylcholine (PC) proceed by similar mechanisms, irrespective of whether the reaction is carried out in homogeneous solution or in water dispersion (*16, 23*). However, little is known about possible medium-dependent changes in the efficiency of a particular antioxidant. We therefore tested the same three compounds in a biologically more relevant system represented by an aqueous dispersion of multilamellar liposomes. In such a system β-carotene, bilirubin, and α-tocopherol at 10 μ*M* inhibited the initial rate of oxidation of soybean PC in air by 22, 87, and 99%, respectively (Fig. 2C). Similar to the homogeneous solution the peroxyl radical trapping activity of bilirubin and β-carotene further increased when the reaction was performed under 2% oxygen. Most important, under these conditions the antioxidant activity of bilirubin surpassed that of α-tocopherol (Fig. 2D).

The reasons for the much higher antioxidant activity of bilirubin in the liposome system in comparison to that observed in homogeneous solution are not known at present. A number of factors have to be considered. Because of the high viscosity of the bilayer, the efficiency of radical production by the lipid-soluble AMVN in liposomes is low (*23*). We therefore raised the temperature from 37° to 50°C (*24*) for the liposome experiments. The concomitant, temperature-dependent, 20% decrease in solubility of oxygen is, however, more than compensated by the higher solubility of oxygen in the bilayer when compared to that in chloroform (*25*). Therefore, differences in the overall concentration of oxygen as a result of the different experimental conditions used in Fig. 2, A and C, are unlikely to explain the much higher peroxyl radical trapping activity of bilirubin in the liposome system. The ability of bilirubin to interrupt the radical chain reaction may be influenced significantly by the probability of interaction between a peroxyl radical and bilirubin. The high lipophilicity of the bilirubin molecule is expected to make the reactive hydrogen atom at C-10 readily accessible to juxtaposed lipid peroxyl radicals formed during lipid oxidation. In contrast, formation of a hydrogen bond between the α-tocopherol chromanol nucleus hydroxyl and the phosphate oxygen of phospholipids (*26*) may partly reduce the accessibility of the antioxidative functional hydroxyl group of the vitamin to the fatty acid acyl chains of a membrane bilayer. Therefore, different relative antioxidant activities under low oxygen concentration, together with differences in the location and mobility of bilirubin and α-tocopherol within membrane bilayers, may explain why bilirubin at 2% oxygen inhibits lipid oxidation in the liposome system at least as efficiently as α-tocopherol.

In birds, amphibians, and reptiles the biliverdin produced in the first step of heme degradation is excreted directly and not further reduced to bilirubin (*13, 27*). Biliverdin could thus play a role as a hydrophilic antioxidant in these creatures. In mammals the polar, and thus presumably nontoxic, biliverdin is reduced in a highly specific NADPH-requiring reaction to form the potentially toxic, nonpolar bilirubin (NADPH, reduced nicotinamide adenine dinucleotide phosphate). Bilirubin then has to undergo further energetically expensive conjugation reactions before it is secreted into bile (*13, 27*). The purpose of biliverdin reduction in mammals has been obscure. Bilirubin associates strongly with albumin and therefore is distributed throughout the entire blood circulation and extravascular space (*13*). Furthermore, free bilirubin has been shown to interact with purified plasma membranes and microsomes with a partition coefficient of about 10^4 (*13, 28*). In view of these observations and the results presented in this report we suggest that one "beneficial" role of bilirubin may be to act as a powerful biological chain-breaking antioxidant. Further studies on the physiological importance of bilirubin are clearly indicated.

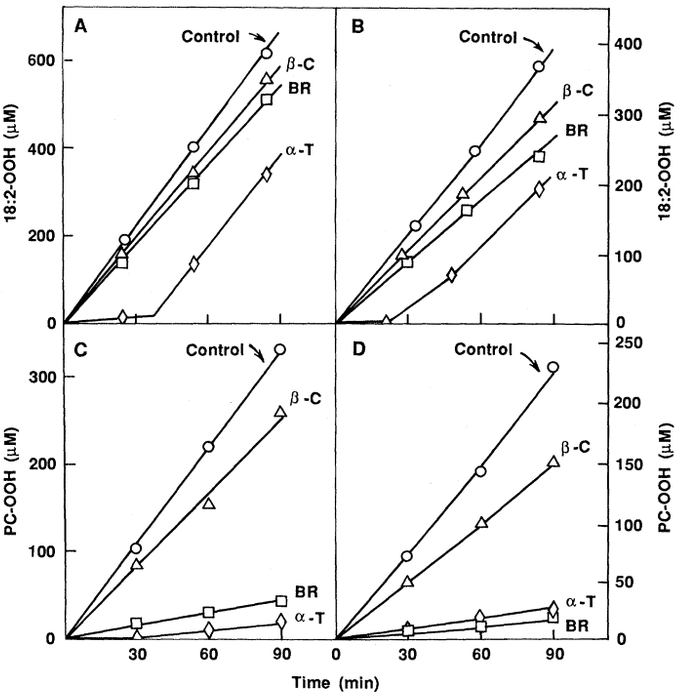

Fig. 2. Comparison of the rate of oxidation of (**A** and **B**) purified linoleic acid (158 m*M*) in homogeneous solution and (**C** and **D**) purified soybean phosphatidylcholine (PC; 10 m*M*) (*29*) in aqueous dispersion under (A and C) air and (B and D) 2% O_2 and 98% N_2 (v/v); in the absence (○) or presence of 10 μ*M* of each, trans-β-carotene (Sigma, type I) (β-C; △), recrystallized bilirubin (BR; □) and *dl*-α-tocopherol (Supelco) (α-T; ◇). The experimental details for (A) and (B) were as described in the legend of Fig. 1. (C and D) Multilamellar liposomes in water were prepared as described by Niki *et al.* (*6*) with the use of purified soybean PC (10 m*M*) and AMVN (0.25 m*M*). The reaction mixture was agitated in a water bath set at 50°C and at various time points aliquots were removed and analyzed for PC hydroperoxides (PC-OOH) at 234 nm by HPLC on an LC-NH_2 column (Supelco) with methanol and 40 m*M* NaH_2PO_4 (9/1, v/v) (1.5 ml/min) as the mobile phase (*29*). The results shown represent the averages of two to four independent experiments, with a variance of less than 7%.

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31. The observed lag in Fig. 1D is thought to reflect the gradual equilibration of the nitrogen atmosphere in the reaction tubes with air over the course of the experiment.
32. The impetus for this study was to provide a test of a general hypothesis put forward by one of us (A.N.G.) that end products of degradative metabolic pathways may play important roles as protective agents, and, that in this context, bilirubin is a likely candidate for such a role. This work was supported by National Cancer Institute Outstanding Investigator grant CA 39910 (B.N.A.), NIEHS (National Institute of Environmental Health Sciences) Center grant ES 01896 (B.N.A.), NIH grants AM 26307, MD 20551 (A.F.M.), and GM 28994 (A.N.G.), NSF grant DMB 85-18066 (A.N.G.), and University of California Toxic Substances Research and Teaching Program (Y.Y.). We also thank H. Hurd for help on earlier aspects of this study.

22 September 1986; accepted 6 January 1987

Construction of a General Human Chromosome Jumping Library, with Application to Cystic Fibrosis

FRANCIS S. COLLINS, MITCHELL L. DRUMM, JEFFERY L. COLE, WENDY K. LOCKWOOD, GEORGE F. VANDE WOUDE, MICHAEL C. IANNUZZI

In many genetic disorders, the responsible gene and its protein product are unknown. The technique known as "reverse genetics," in which chromosomal map positions and genetically linked DNA markers are used to identify and clone such genes, is complicated by the fact that the molecular distances from the closest DNA markers to the gene itself are often too large to traverse by standard cloning techniques. To address this situation, a general human chromosome jumping library was constructed that allows the cloning of DNA sequences approximately 100 kilobases away from any starting point in genomic DNA. As an illustration of its usefulness, this library was searched for a jumping clone, starting at the *met* oncogene, which is a marker tightly linked to the cystic fibrosis gene that is located on human chromosome 7. Mapping of the new genomic fragment by pulsed field gel electrophoresis confirmed that it resides on chromosome 7 within 240 kilobases downstream of the *met* gene. The use of chromosome jumping should now be applicable to any genetic locus for which a closely linked DNA marker is available.

THE USE OF LINKAGE ANALYSIS (*1*) and high-resolution cytogenetics has allowed chromosomal mapping in an increasing number of disorders of single human genes, even in situations where gene function is unknown. Recent examples include Huntington disease (*2*), adult polycystic kidney disease (*3*), cystic fibrosis (*4–7*), chronic granulomatous disease (*8*), Duchenne muscular dystrophy (*9*), and familial retinoblastoma (*10*). In the last three of these disorders mapping has led directly to the cloning of a candidate gene, raising hopes that this "reverse genetics" (*11*) approach may be widely successful for cloning disease-associated genes for which no protein product is known. In general, however, the closest DNA markers to a disease gene are often hundreds of kilobases away. In cystic fibrosis (CF), for example, the gene has been localized to the long arm of chromosome 7 by the finding that it is closely linked to two molecular probes, the *met* oncogene (*6*) and the DNA fragment pJ3.11 (*7*), both of which are placed at less than 0.5 centimorgan from the CF gene by current linkage estimates (*12*). Because, on the average, 1 centimorgan is represented by about 1000 kilobases (*13*), cloning the CF locus or any other disease locus by reverse genetics is likely to require crossing hundreds of kilobases. To address the general problem of cloning over such large distances, we have recently described a method of "chromosome jumping" (*13, 14*). In a model system we showed that this technique could be used to cross a distance of 45 kb (*14*). Lehrach and co-workers have independently described a similar scheme (*15*). To demonstrate the practical application of our technique, we have constructed a general jumping library and derived from it a cloned sequence of DNA that lies approximately 100 kb downstream from the *met* oncogene.

A human chromosome jumping library was constructed (*16*) from human lymphoblastoid cell DNA (see Fig. 1). In this instance, the distance of the jumps represented in the library (the "hopsize") was chosen to be approximately 100 kb by size selection of genomic DNA molecules in the size range of 80 to 130 kb. The principle of the technique, as previously outlined (*13–15*), depends on the formation of large genomic circles from size-selected DNA, bringing together the genomic fragments that were originally 100 kb apart. Note that a light partial digestion with the restriction enzyme Mbo I, which cuts frequently in genomic DNA, is performed prior to size selection, so that there is no significant bias for a particular sequence to occur at the junction of the circles. In each clone, the position of the joining of the two genomic fragments is marked by a suppressor transfer RNA or supF gene (*17*), which allows selection for these fragments after the circles are digested with Eco RI and ligated into a

F. S. Collins, Departments of Internal Medicine and Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109.
M. L. Drumm, Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109.
J. L. Cole, W. K. Lockwood, M. C. Iannuzzi, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109.
G. F. Vande Woude, National Cancer Institute–Frederick Cancer Research Facility, Frederick, MD 21701.