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Response to Adriamycin of Transplasma Membrane Electron Transport in Adriamycin-Resistant and Nonresistant HL-60 Cells

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Adriamycin, 10^{-8}–10^{-5} M, inhibited transplasma membrane electron transport of uninduced HL-60 cells susceptible to adriamycin and not in uninduced HL-60 cells resistant to adriamycin as measured by reduction of external ascorbate free radical. Electron flow across the plasma membrane was measured with the intact living cells by means of a simple assay procedure whereby the transported electrons were captured by ascorbate free radical to slow the rate of chemical oxidation of ascorbate. The response to adriamycin was rapid with maximum inhibition in less than 1 min. Preincubation was not required and the inhibition presumably was not mediated through effects on DNA replication or transcription. Except at the highest concentration tested of 10 μM, both transplasma membrane electron transport and growth were unaffected by adriamycin with a line of HeLa cells resistant to the drug. The findings provide evidence, using a physiological acceptor, ascorbate free radical, for a direct inhibition of transmembrane electron transport of HL-60 cells by adriamycin that correlates closely with adriamycin inhibition of cell growth. The lack of response with resistant cells suggests an alternative mechanism for adriamycin resistance not necessarily based on transport control.

KEY WORDS: Adriamycin; ascorbate; electron transport; growth; plasma membrane (HL-60 cells).

INTRODUCTION

Transplasma membrane electron transport is a characteristic ubiquitous to eukaryotic cells (Crane *et al.*, 1990). The most extensively demonstrated activity is a NADH-acceptor oxido-reductase seen with cells where impermeant iron compounds such as ferricyanide serve as acceptors (Crane *et al.*, 1985). Using reduction of external iron compounds, Crane *et al.* (1980) reported inhibition of plasma membrane electron transport by adriamycin. The inhibition of electron transport by adriamycin was more pronounced in transformed cell lines than in nontransformed cell lines (Sun *et al.*, 1983, 1986).

As an alternative to the reduction of external iron compounds, most of which are nonphysiological (Crane *et al.*, 1985), we have employed the ascorbate free radical generated from ascorbate under physiological conditions as acceptor (Navas *et al.*, 1992). The assay is based on the ability of transported electrons to reduce ascorbate free radical and thereby slow the rate of external ascorbate oxidation monitored spectrophotometrically. Using this assay, our findings show inhibition of electron transport and growth of uninduced human leukemia (HL-60) cells by adriamycin. Alternatively, neither the inhibition of cell growth nor the inhibition of electron transport is seen with a line of HL-60 cells resistant to adriamycin. These findings may provide the basis for an alternative mechanism to adriamycin resistance not based on transport control.

MATERIAL AND METHODS

HL-60 cells were cultured in growth medium consisting of RPMI 1640 with 10% heat-inactivated

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fetal calf serum supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B, at 37°C for 4 days in a humidified atmosphere of 5% CO_2 and 95% air (Alcain *et al.*, 1991). The cells were inoculated at 5×10^6 cells/ml. Cells were concentrated from stock cultures by centrifugation at 1000 *g* and washed twice with serum-free RPMI-1640 medium and resuspended for assay in 100 mM Tris-HCL, pH 7.4.

Transplasma membrane electron transport was estimated from the reduction of ascorbate free radical (slowing of ascorbate oxidation) measured at 265 nm (Winkler, 1987). Ascorbate was monitored continuously with time using a Shimadzu UV-160 spectrophotometer over 10 min. Approximately 10^6 cells, suspended in freshly prepared 0.1 M Tris-HCl buffer, pH 7.4, containing 0.2 mM ascorbate, were used per assay in a final volume of 3 ml. Cells were preincubated at 37°C for 5 min prior to resuspension in buffered ascorbate with or without adriamycin at 37°C. An extinction coefficient for ascorbate determined for Tris-HCl buffer at pH 7.4 of 11.2 mM^{-1} cm^{-1} (Alcain *et al.*, 1991) was used.

Adriamycin solutions were freshly prepared in distilled water and protected from light. Control preparations received an equivalent amount of distilled water and were assayed in parallel.

RESULTS

The assay conditions described measured a linear decrease in absorbance at 265 nm in the presence of ascorbate as a result of the autoxidation of ascorbate in air. Within the linear range of the assay (0.2 mM ascorbate, 2 to 5×10^5 cells per ml), the rate of ascorbate oxidation was slowed by the addition of cells in proportion to the number of cells present (Alcain *et al.*, 1991; Navas *et al.*, 1992). Boiled cells were without activity, and plasma membranes isolated from the cells also demonstrated an ability to reduce ascorbate free radical. However, with the isolated plasma membranes, it was necessary to first add exogenous NADH as a source of electrons. As with cells, boiled plasma membranes were inactive as were plasma membranes without NADH (Alcain *et al.*, 1991; Navas *et al.*, 1992).

The electron flow in uninduced HL-60 cells as measured by a slowing of ascorbate oxidation (ascorbate radical reduction) was inhibited in a dose-dependent manner by adriamycin (Fig. 1, lower curve). Inhibition was maximal at about 1 μM where a reduction of approximately 50% in electron flow was observed. With HL-60 cells resistant to adriamycin, the response of electron flow to adriamycin was insignificant (Fig. 1, upper curve).

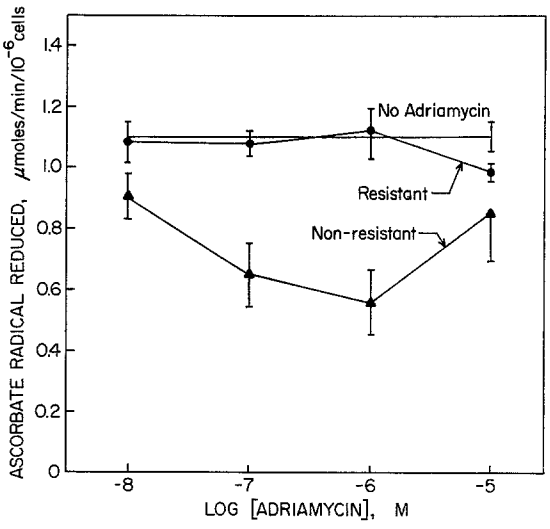

Fig. 1. Dose response of transplasma membrane electron transport as measured by reduction of ascorbate free radical of nonresistant HL-60 cells (filled triangles) and HL-60 cells resistant to adriamycin (filled circles). Results are means from four experiments with duplicate or triplicate determinations ± standard deviations among experiments. The rates reported are the differences between cells with or without adriamycin and no cells present (ascorbate alone). In this series of experiments both the resistant and nonresistant cells exhibited the same initial radical reduction rates (no adriamycin).

The response to adriamycin with nonresistant cells also had the appearance of being bimodal (Fig. 1). At 10 μM adriamycin, ascorbate oxidation was less inhibited in the presence of adriamycin than at 1 μM.

The HL-60 cells used in this study exhibited a differential growth response to adriamycin similar to that seen with electron transport (Fig. 2). The uninduced cells were strongly inhibited by adriamycin over the concentration ranges 10^{-8}–10^{-5} M (Fig. 2B). Maximum inhibition was observed at 1 μM adriamycin where the cell population was reduced by approximately 80%. In contrast, the growth of HL-60 cells resistant to adriamycin was largely unaffected by adriamycin (Fig. 2A) except at the highest adriamycin concentration tested of 10 μM, as was plasma membrane electron transport (Fig. 1, upper curve).

With uninduced HL-60 cells, the response to adriamycin was rapid, as seen by the slowing of ascorbate oxidation (Fig. 3). The latter was used as a measure of ascorbate radical reduction. With no

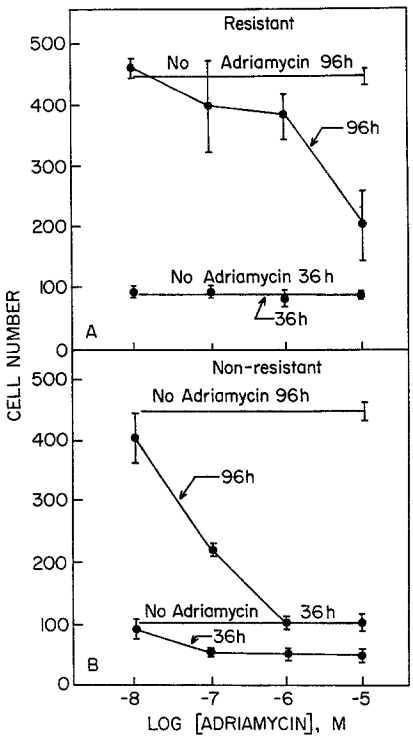

Fig. 2. Dose response to adriamycin of cell number after 36 and 96 h with HL-60 cells resistant to adriamycin (A) and with normal HL-60 cells susceptible to adriamycin (B). Results are averages of four independent experiments ± standard deviations.

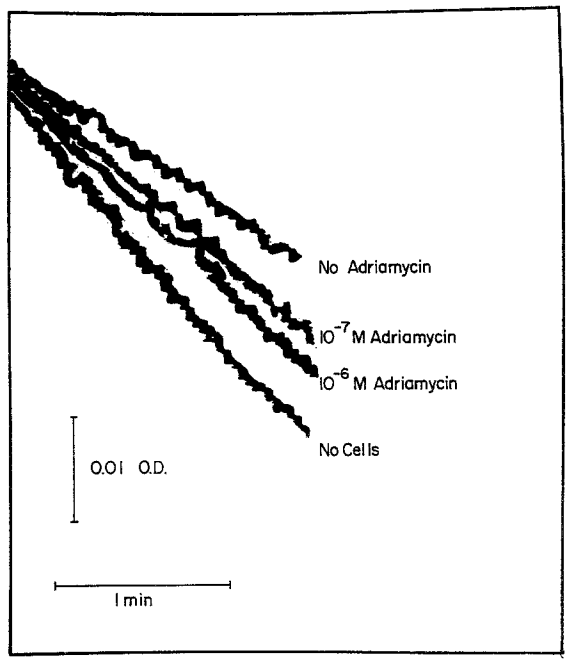

Fig. 3. Kinetics of slowing of ascorbate oxidation as a measure of transmembrane electron transport and inhibition by 10^{-6} M adriamycin with HL-60 cells susceptible to adriamycin. The change in absorbance at 265 nm was measured about 10^6 cells. Adriamycin was added at $t = 0$. Inhibition occurred rapidly and without perceptible lag.

cells present, a linear decrease in absorbance at 265 nm was observed (no cells). With added cells and no adriamycin, the rate of ascorbate oxidation was slowed, the difference being proportional to the rate of reduction of ascorbate free radical. With added adriamycin, the rate of ascorbate oxidation was partially restored (rate of ascorbate free radical was slowed), indicative of a reduction of electron flow by adriamycin. The inhibition occurred rapidly and without perceptible lag. Similarly, the degree of inhibition of electron transport by adriamycin was independent of time of preincubation (Fig. 4) with both susceptible and resistant HL-60 cells. With the nonresistant cells, the same degree of inhibition was observed with 30 min preincubation as with no preincubation. For the adriamycin-resistant cells, no significant inhibition of electron transport was observed with or without preincubation.

Since iron in solution or in chelates may catalyze oxidation of ascorbate and since iron can be released from cells (Thorstensen and Romslø, 1990), a series of controls were carried out to determine if the observed

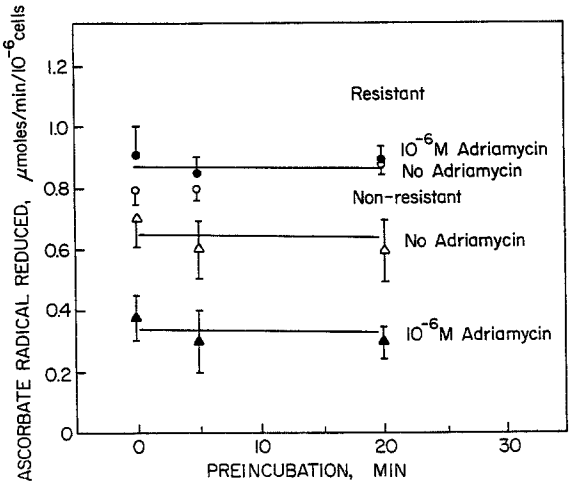

Fig. 4. Adriamycin inhibition of electron transport is independent of time of preincubation (0–2 min) comparing resistant (circles) and nonresistant (triangles) cells in the presence (solid symbols) or absence (open symbols) of 10^{-6} M adriamycin. Results are means from three determinations in each of three experiments ± standard deviations among experiments.

inhibitions were the result of adriamycin acting as a chelator of released iron. In these experiments, cells were treated with adriamycin either in the presence or absence of ascorbate, and supernatants were tested for activity after removal of cells by centrifugation. Additionally, cell supernatants were prepared and adriamycin was added.

Addition of the supernatants to the ascorbate mixture in every instance retarded rather than accelerated the rate of ascorbate oxidation. Additionally, addition of adriamycin was without effect either on supernatants already prepared or when added to cells (either with or without incubation for 2 min) followed by removal of cells by centrifugation. These results were obtained under conditions of treatment (e.g., 1 μM adriamycin) where the normal response to adriamycin was observed in the same series of determinations with cells present. Adriamycin was without effect on the supernatant fractions from either resistant or nonresistant cells. No change was observed even with cells present in the absence of ascorbate, suggesting that contributions of substances other than ascorbate to absorbance changes at 265 nm were minimal.

DISCUSSION

Adriamycin is among the most used antitumor drugs (Arcamone, 1985). Its mode of action has been presumed widely to involve interaction with DNA (Cullianane and Phillips, 1990) despite reports of impermeant adriamycin conjugates and cytotoxicity against tumor cells that may be greater or equal to that of the parent drug (Tritton and Yee, 1982; Rogers and Tokes, 1984; Yeh and Faulk, 1984). It has also been proposed to generate free radicals (Bachur *et al.*, 1979; Powis, 1989; Algeria *et al.*, 1990) and to inhibit specific enzymes (Sun and Crane, 1984; Faulk *et al.*, 1988; Sun *et al.*, 1992). Its effectiveness is decreased by development of cellular resistance based on mechanisms which include active extrusion, decreased uptake, or increased detoxification (Tewey *et al.*, 1984; Crane *et al.*, 1985; Sun *et al.*, 1987). In this regard, observations that adriamycin can affect functions in the plasma membrane in addition to interaction with DNA (Solie and Yuncker, 1978; Tokes *et al.*, 1982; Burke and Tritton, 1985; Patrick, 1988; Yokoyama *et al.*, 1990; Vichi and Tritton, 1992) have added significance.

The plasma membrane electron transport system may be coupled to growth or growth control. Uncontrolled growth of transformed cells is very sensitive to adriamycin and other active anthracycline compounds (Sun and Crane, 1988, 1990). Inhibitions of transmembrane electron transport by adriamycin are observed within the first minute of addition of drug and are presumed to be direct since more than 90 min are normally required for adriamycin to reach 1/2 maximum concentration in the nucleus. Also the adriamycin–transferrin conjugates, which are 10 times more active than adriamycin in growth inhibition, do not introduce adriamycin into the nucleus of viable cells since there is no quenching of adriamycin fluorescence (Barabas *et al.*, 1991). Inhibition of NADH oxidase activity in isolated plasma membranes also shows direct action of the drug at the plasma membrane.

Transfer of electrons from internal electron donors to external impermeant electron acceptors, such as ferric iron associated with ferricyanide or diferric transferrin, was used by Sun and Crane (1984) as a measure of plasma membrane electron transport to demonstrate adriamycin inhibition. This inhibition also was observed with adriamycin conjugated to diferric transferrin but at one-tenth the concentration required for inhibition by unconjugated adriamycin.

The targeting of adriamycin to actively growing cells which have increased expression of transferrin receptors by means of transferrin–adriamycin conjugates was introduced by Faulk *et al.*, (1980). These conjugates of adriamycin and diferric transferrin with glutaraldehyde were therapeutic in treatment of leukemia (Yeh *et al.*, 1984; Faulk *et al.*, 1990a) and inhibited growth of both transformed and adriamycin-resistant cells (Yeh *et al.*, 1984; Faulk *et al.*, 1988, 1990a, b). The conjugates proved to be disproportionately more effective compared to free adriamycin than would be anticipated solely on the basis of increased numbers of transferrin receptors. However, other forms of conjugated adriamycin, i.e., to a monoclonal anti-transferrin receptor antibody, may be less effective than the free drug or require hydrolysis for activity (Faulk *et al.*, 1990b).

Growth of RLA209-15 fetal rat liver cells and RPNA209-1 rat pineal gland cells at 33°C (transformed phenotype) show a much slower rate of diferric transferrin reduction and ferricyanide reduction (Sun *et al.*, 1986) in both the fast and slow phase than do the cells which have been grown at 40°C (nontransformed phenotype). Moreover, at 33°C the

cultures show a rate of growth about one-third of that at 40°C. A similar difference has been observed with SV40 transformed-3T3 and nontransformed-3T3 cells (Crane *et al.*, 1985). Hepatoma cells have a rate of ferricyanide reduction 30% less in the fast phase and 60% less in the slow phase than isolated fetal liver cells (Sun *et al.*, 1983). High rates of ferricyanide reduction have also been observed with adult liver cells (Clark *et al.*, 1981). This higher transmembrane redox activity of nontransformed cells also shows less sensitivity to anthracycline compounds than does that of transformed cells (Sun *et al.*, 1983) and, together with selective drug inhibition of transformed cells, is further evidence for a change in their redox enzymes. Alteration of V_{max} and K_m of the enzyme has been described as well (Sun *et al.*, 1986). These findings have been interpreted as a basis for understanding selective effects of these antitumor drugs on cancer cells (Sun and Crane, 1988, 1990).

With nonresistant HL-60 cells and HL-60 cells resistant to adriamycin, a differential response to growth and plasma membrane electron transport was observed in the present study that paralleled that of the comparisons of tumor cells and their more normal counterparts. Here transplasma membrane electron transport was measured by the slowing of ascorbate autoxidation (Navas *et al.*, 1992), and results were similar to those obtained using impermeant iron compounds (Crane *et al.*, 1980). An apparent tendency toward a bimodal response of the NADH oxidase of nonresistant cells was observed for which we have no explanation. However, inhibition of both growth and ascorbate radical reduction was observed with both resistant and nonresistant cells at 10 μM adriamycin (Figs. 1 and 2).

Growth of nonresistant HL-60 cells was susceptible to inhibition by adriamycin as was transplasma membrane electron transport much like that of transformed cells. In contrast, both growth and transplasma membrane electron transport of HL-60 cells resistant to adriamycin was largely unaffected by adriamycin except at extremely high concentrations. These findings suggest that some component of the electron transport chain may be susceptible to adriamycin inhibition and that in the transformed cells the adriamycin-sensitive form of the activity is constitutively expressed. Similar conclusions were reached from measurements of growth-factor-responsive NADH oxidase activities of plasma membrane of normal liver and of preneoplastic liver nodules induced by feeding animals the carcinogen *N*-acetylaminofluorene (Morré *et al.*, 1991).

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REFERENCES

Alcain, F. J., Burón, M. I., Villalba, J. M., and Navas, P. (1991). *Biochem. Biophys. Acta* **1073,** 380–385.

Algeria, A. E., Rodriguez, M. S., and Hernandez, J. (1990). *Biochim. Biophys. Acta* **1035,** 51–55.

Arcamone, F. (1985). *Cancer Res.* **45,** 5995–5999.

Bachur, H. R., Gordon, S. L., Gee, M. V., and Kon, H. (1979) *Proc. Natl. Acad. Sci.* **76,** 945–957.

Barabas, K., Sizensky, J. A. and Faulk, W. P. (1991). *Am. J. Reprod. Immunol.* **25,** 120–124.

Burke, T. G., and Tritton, T. R. (1985). *Biochemistry* **24,** 5972–5980.

Clark, M. G., Patrick, E. J., Patten, G. S., Crane, F. L., Löw, H., and Grebing, C. (1981). *Biochem. J.* **200,** 565–572.

Crane, F. L., Crane, H. E., Sun, I. L., MacKellar, W. C., Grebing, C., and Löw, H. (1980). *Biochem. Biophys. Res. Commun.* **93,** 746–754.

Crane, F. L., Sun, I. L., Clark, M. G., Grebing, C., and Löw, H. (1985). *Biochim. Biophys. Acta* **811,** 233–264.

Crane, F. L., Löw, H., and Morré, D. J. (1990). In *Oxidoreduction at the Plasma Membrane: Relation to Growth and Transport* (Crane, F. L., Morré D. J., and Löw, H., eds.), C.R.C. Press, Boca Raton, Florida. Vol. 1, pp. 1–27.

Cullianane, C., and Phillips, D. R. (1990). *Biochemistry* **29,** 5638–5645.

Faulk, W. P., Hsi, B. L., and Stevens, P. J. (1980). *Lancet* **2,** 390–392.

Faulk, W. P., Torry, D. S., Harats, H., McIntyre, J. A., and Taylor, C. G. (1988). In *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Crane, F. L., Morré, D. J., and Löw, H. eds.), Plenum, New York, pp. 173–180.

Faulk, W. P., Taylor, C. G., Yeh, C. J. G., and McIntyre, J. A. (1990a). *Mol. Biotherm.* **2,** 57–60.

Faulk, W. P., Harats, H., and Berczi, A. (1990b). In *Oxidoreduction at the Plasma Membrane: Relation to Growth and Transport* Crane, F. L., Morré D. J., and Löw, H., eds.), CRC Press, Boca Raton, pp. 205–224.

Morré, D. J., Crane, F. L., Eriksson, L. C., Löw, H., and Morré, D. M. (1991). *Biochim. Biophys. Acta* **1057,** 140–146.

Navas, P., Alcain, F., Burón, M. I., Rodriquez-Aguilera, J. C., Villalba, J. M., Morré, D. M., and Morré, D. J. (1992). *FEBS Lett.* **299,** 223–226.

Patrick, D. (1988). *Cancer Invest.* **6,** 439–451.

Powis, G. (1989). *Free Radical Biol. Med.* **6,** 63–101.

Rogers, K. E., and Tokes, Z. A. (1984). *Biochem. Pharmacol.* **33,** 605–608.

Solie, T. N., and Yuncker, C. (1978). *Life Sci.* **22,** 1907–1920.

Sun, I. L., and Crane, F. L. (1984). *Proc. Indiana Acad. Sci.* **93,** 267–274.

Sun, I. L., and Crane, F. L. (1988). In *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Crane, F. L., Morré, D. J., and Löw, H., eds.), Plenum Press, New York, pp. 181–190.

Sun, I. L., and Crane, F. L. (1990). In *Oxidoreduction at the Plasma Membrane: Relations to Growth and Transport, Vol. I, Animals* (Crane, F. L., Morré, D. J., and Löw, H., eds.), CRC Press, Boca Raton, pp. 257–280.

Sun, I. L., Crane, F. L., Chou, J. Y., Löw, H., and Grebing, C. (1983). *Biochem. Biophys. Res. Commun.* **116,** 649–654.

Sun, I. L., Crane, F. L., and Chou, J. Y. (1986). *Biochim. Biophys. Acta* **886,** 327–336.

Sun, I. L., Navas, P., Crane, F. L., Morré, D. J., and Löw, H. (1987). *Biochem. Int.* **14,** 119–127.

Sun, I. L., Sun, E. E., Crane, F. L., Morré, D. J., Lindgren, A., and Löw, H. (1992) *Proc. Natl. Acad. Sci.* **89,** 11126–11130.

Tewey, K. M., Rowe, T. C., Yand, I., Halligan, B. D., and Liu, L. F. (1984). *Science* **266,** 466–468.

Thorstensen, K., and Romslø, I. (1990). *Biochem. J.* **271,** 1–10.

Tokes, Z. A., Rogers, K. E., and Rembaum, A. (1982). *Proc. Natl. Acad. Sci.* **79,** 2026–2030.

Tritton, T. R., and Yee, G. (1982). *Science* **217,** 248–250.

Vichi, P., and Tritton, T. R. (1992). *Cancer Res.* **52,** 4135–4138.

Winkler, B. S. (1987). *Biochim. Biophys. Acta* **925,** 258–264.

Yeh, C. J., and Faulk, W. P. (1984). *Clin. Immunol. Immunopathol.* **32,** 1–11.

Yeh, C. J. G., Taylor, C. G., and Faulk, W. P. (1984). *Proteids Biol. Fluids* **32,** 441–444.

Yokayama, M., Miyauchi, M., Yamada, N., Okano, T., Sakarai, Y., Kataoka, K., and Inoue, S. (1990). *Cancer Res.* **50,** 1963–1970.