of the clear supernatant was layered on a linear 5 to 20 percent sucrose gradient of 5 ml and centrifuged at 148,000*g* for 20 hours. The gradients were fractionated by piercing the bottom of the tube and collecting 0.25-ml fractions. Radioactivity in each fraction was determined by liquid scintillation counting, as described earlier (*5*).

From our previous work with rat tissue, the presence of the binding protein is indicated by a peak of radioactivity in the 2*S* region of the gradient, which is reduced when unlabeled retinoic acid has been added. A second peak at 4.6*S* is frequently observed and, in contrast to the 2*S* peak, this 4.6*S* binding is not diminished by excess unlabeled retinoic acid. It appears to be nonspecific binding to serum albumin which contaminates most preparations (*10*).

As shown in Fig. 1, a peak of radioactivity sedimenting in the 2*S* region of the gradient was observed for malignant tissue from human lung and breast. Normal tissue from the same organ did not show the specific binding protein at 2*S* but only the nonspecific binding at 4.6*S*. A total of four carcinomas have been examined (one lung and three breast) and all showed 2*S* binding indicative of the retinoic acid binding protein.

The human retinoic acid binding protein detected in these tumors is specific for retinoic acid as binding is diminished by excess all-*trans*-retinoic acid only, but not by retinol, retinal, or oleic acid. The molecular weight of this protein is approximately 15,000 daltons, when calculated from the sedimentation data by the method of Martin and Ames (*11*). Gradients with standard proteins were treated in parallel for calibration.

While we did not detect the retinoic acid binding protein in normal breast and lung tissue, we have observed its presence in the human uterus (*12*). This is similar to the situation in the rat, in that the lung of the adult rat shows no evidence for the binding protein but it is present in the uterus (*6*). However the cellular retinoic acid binding protein is clearly evident in rat fetal lung (Fig. 1) at day 19 of gestation. Currently it is not possible for us to extend our observations to human fetal material, but the presence of the retinoic acid binding protein in malignant tissue may be another case of the occurrence of a cellular component in malignant tissue which normally is observed only at the fetal stage, as has been reported for some other proteins (*13*).

There is considerable evidence, recently reviewed (*14*), that vitamin A status or administration of retinol, retinoic acid, or their derivatives affects the development of some tumors (*15*). Recently Bollag (*16*) showed that administration of an aromatic analog of retinoic acid leads to a marked regression of carcinogen-induced established skin papillomas and carcinomas of mice. However, the compound has no significant effect on transplantable tumors examined, including Ehrlich ascites carcinoma. It is of interest that we find no detectable retinoic acid binding protein in this carcinoma (*17*). The presence of the binding protein may be required in order for such an antimetabolite of retinoic acid to have a therapeutic effect.

The observations reported here were made with a limited number of human carcinomas which all contained a high proportion of epithelial cells. Since in the samples of normal tissue there was also a considerable amount of these cells (*8*), the presence of the retinoic acid binding protein represents a difference between malignant and normal cells rather than a drastic change in epithelial content. The occurrence of a cellular retinoic acid binding protein indicates that at least in some malignant tissues there is an altered interaction of retinoic acid within the cell compared to normal tissue.

DAVID E. ONG
*Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232*
DAVID L. PAGE
*Department of Pathology, Vanderbilt University School of Medicine*
FRANK CHYTIL
*Departments of Biochemistry* and *Medicine, Vanderbilt University School of Medicine*

**References and Notes**

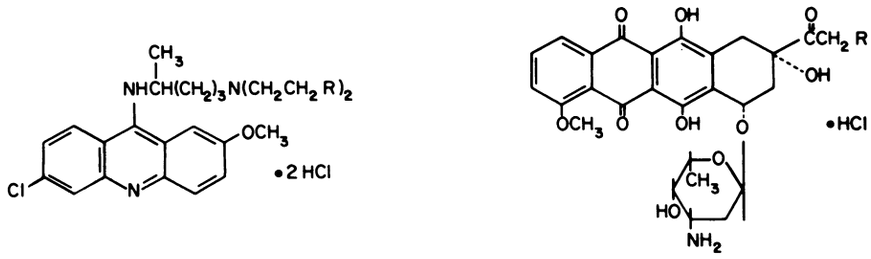
1. S. B. Wolbach and P. R. Howe, *J. Exp. Med.* **42**, 753 (1925).
2. R. J. Emerick, M. Zile, H. F. DeLuca, *Biochem. J.* **102**, 606 (1967).
3. J. E. Dowling and G. Wald, *Proc. Natl. Acad. Sci. U.S.A.* **46**, 587 (1960).
4. J. N. Thompson, J. McHowell, G. A. J. Pitt, *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **159**, 510 (1964).
5. M. M. Bashor, D. O. Toft, F. Chytil, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3483 (1973).
6. D. E. Ong and F. Chytil, *J. Biol. Chem.* **250**, 6113 (1975).
7. ———, *Nature (Lond.)* **255**, 74 (1975).
8. The breast carcinomas were all of the infiltrating duct type with demonstrated metastasis to axillary lymph nodes in each case. The lung carcinoma was of the nonkeratinizing epidermoid type. Histologic examination of each analyzed sample demonstrated a great amount of interstitial collagen formation in one of the breast carcinomas and a normal component of epithelial elements in each of the samples of normal tissue. Special effort was made to exclude fat from the breast samples and bronchial elements from the normal lung samples, although the lung sample contained bronchiolar elements and normal alveolar elements. Histologic examination of the relative numbers of epithelial cells indicated approximately 75 percent in the lung tumor, whereas the normal lung tissue contained 50 percent epithelial cells. Breast tumors ranged from 80 to 95 percent epithelial cells while normal samples were 50 to 85 percent.
9. S. G. Korenman, *J. Clin. Endocrinol.* **18**, 127 (1968).
10. M. M. Bashor, thesis, Vanderbilt University (1974).
11. R. Martin and B. Ames, *J. Biol. Chem.* **236**, 1372 (1961).
12. F. Chytil, D. L. Page, D. E. Ong, *Int. J. Vitam. Nutr. Res.*, in press.
13. F. Shapira, *Adv. Cancer Res.* **18**, 77 (1973); G. I. Abelev, *ibid.* **14**, 295 (1971).
14. T. H. Maugh II, *Science* **186**, 1198 (1974).
15. E. W. Chu and R. A. Malmgren, *Cancer Res.* **25**, 884 (1965); H. McMichael, *ibid.*, p. 947; V. Saffiotti, R. Montesano, A. R. Sella-Kumar, S. A. Borg, *Cancer* **20**, 857 (1967); D. E. Davies, *Cancer Res.* **27**, 237 (1967); P. M. Newberne and A. E. Rogers, *J. Natl. Cancer Inst.* **50**, 439 (1973); M. V. Nettesheim, *ibid.*, p. 1599.
16. W. Bollag, *Eur. J. Cancer* **10**, 731 (1974).
17. L. S. Hnilica provided the specimen.
18. Supported by PHS grants HD-05384, HL-15341, HL-14214, and CA-16547. We thank the Hoffmann–La Roche Company for radioactive retinoic acid.

28 March 1975; revised 20 May 1975

## Differential Fluorescent Staining of Human Chromosomes with Daunomycin and Adriamycin—The D-Bands

Abstract. *Human chromosome preparations were treated with a group of anthracycline antibiotics. Well-defined, orange-red fluorescent bands were observed on chromosomes after the slide was stained with daunomycin and adriamycin but not with nogalamycin. The characteristic differential bands appeared to be similar to the banding patterns obtained by the quinacrine techniques. Interaction of these antibiotics with DNA could provide information on the general mechanism of fluorescent banding. Further, these bands (D-bands) appeared to be more stable than the Q-bands and may have some usefulness for routine clinical cytogenetic analysis.*

Fluorescent labeling of chromosomes by quinacrine and quinacrine mustard (Fig. 1a) and other DNA-binding fluorochromes has become a well-established technique for the identification and analysis of chromosomes and chromosome regions (*1*). Intercalation is thought to be the primary mode of binding of the fluorochromes to chromosomal DNA (*2*). In the interaction of quinacrine with chromosomes, the banding patterns appear to be derived from the variable quantum efficiency of quinacrine fluorescence as a function of the base composition around the intercalation site (*3*). Recently, it was shown that uninterrupted stretches of A-T (adenine-thymine) base pairs enhance quinacrine fluorescence, while a progressive quenching of quinacrine fluorescence is observed with increasing guanine content of the DNA (*4*). It has also been shown that several factors which affect the quenching or enhancement of fluorescence by native DNA with specific nucleotide sequences corresponds well with the effect of brightness and differentiation of Q-bands on the



a) $R_1$ = H Quinacrine dihydrochloride
$R_2$ = Cl Quinacrine mustard

b) $R_1$ = H Daunomycin
$R_2$ = OH Adriamycin

Fig. 1. Structure of quinacrine (a) and anthracycline antibiotics (b).

fixed chromosomes (*5*). These observations suggest that differential distribution of base composition may play an important role in displaying fluorescent bands. However, other mechanisms such as nonhistone protein-DNA interaction with the fluorochromes may also play an equally important role in producing differentiated banding patterns (*6*).

The interaction of the anthracycline group of antibiotics daunomycin, adriamycin (Fig. 1b), and nogalamycin with DNA has been reported (*7*) to be similar to that of the aminoacridines (quinacrine, proflavine). Anthracycline-caused increases in viscosity and decreases in density of native DNA are consistent with an intercalation binding (*8, 9*). Similar to the fluorescence quenching in the interaction of the aminoacridines with most natural DNA, the anthracyclines' fluorescence is quenched by the interaction with DNA (*8*). Thus, it is important to see whether this group of antibiotics would also produce differential bands on chromosomes. Moreover, the binding of the anthracyclines to DNA is little affected by increases in ionic strength, conditions under which the aminoacridines are fully displaced (*8*). Since the fluorescence of chromosomes bound to quinacrine fades quickly, the use of these anthracyclines as fluorochromes for chromosome banding was investigated.

Well-defined and reproducible orange-red fluorescent banding patterns were observed on human chromosomes after treatment with daunomycin (Cerubidine or Daunorubicine-HCl). Chromosome preparations were obtained from short-term lymphocyte culture by the routine air-drying procedure. The staining solutions consisted of 0.5 mg of Cerubidine per milliliter (obtained from Poulenc Limited, Montreal) in 0.1*M* sodium phosphate buffer with a *p*H of 4.3. Slides were stained for 15 minutes and washed in three changes of phosphate buffer for a total of 6 minutes and mounted. Preparations were observed with a Zeiss WL fluorescent microscope equipped with an ultra-dark field condensor for transmitted illumination. The light source was a d-c powered HBO W/2 mercury burner. A KP-500 filter was used for excitation, and the barrier filter was set at No. 50. The characteristic differential bands on chromosomes obtained by the daunomycin fluorescent technique (D-band) appeared to be similar to Q-bands. The distal long arm of the Y chromosome also fluoresced brightly. Further, the Y-body can also be identified in the interphase nuclei. Variable bright fluorescent regions (bands) were found on the centromeric area of chromosome No. 3 and on the short arm of chromosome No. 13 (Fig. 2).

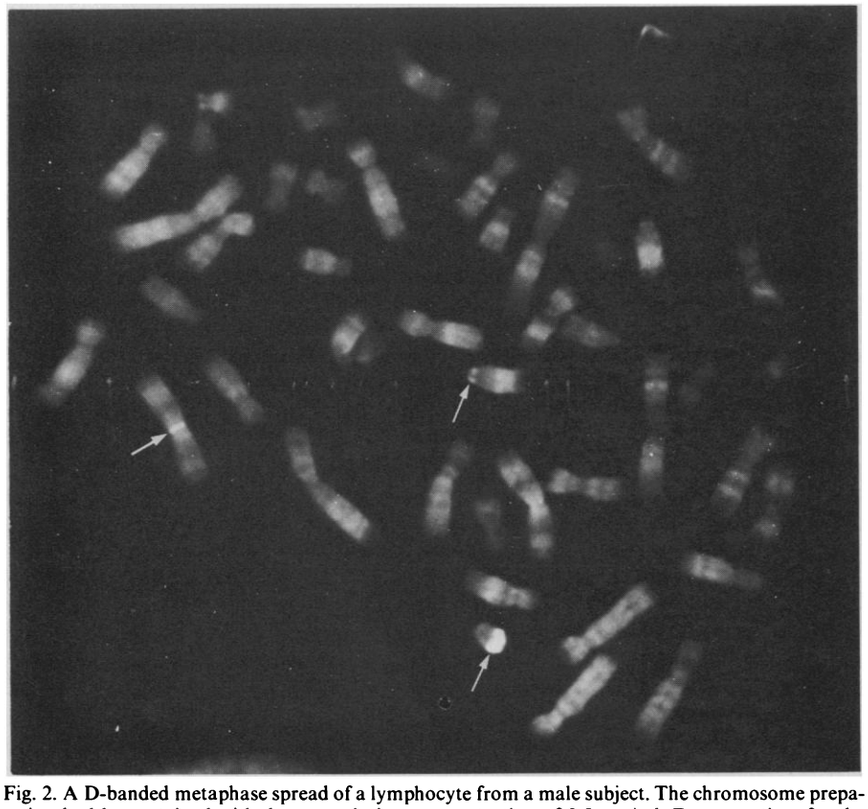


Fig. 2. A D-banded metaphase spread of a lymphocyte from a male subject. The chromosome preparation had been stained with daunomycin in a concentration of 0.5 mg/ml. Exposure time for the negative was 60 seconds, using Kodak high-contrast copy film (ASA = 64). The banding pattern on chromosomes was similar to those produced by quinacrine fluorescent technique. A brilliant, bright fluorescent area of the distal long arm of the Y chromosome and the variable bright fluorescent bands on chromosomes 3 and 13 are also readily seen (indicated by arrows).

Another anthracycline antibiotic, adriamycin (Doxorubicin-HCl), which is closely related to daunomycin (Fig. 1b), was also found to produce banding patterns on chromosomes like those obtained by the daunomycin. The concentration of Doxorubicin used to stain the slide was 0.2 mg/ml; the rest of the staining procedure was essentially the same as that for daunomycin.

A third antibiotic of the anthracycline group, nogalamycin, was used as a fluorochrome for chromosome banding. In contrast to daunomycin and adriamycin, the nogalamycin did not produce clear fluorescent bands, and the overall fluorescence on the chromosome was relatively dull. It has been shown that the aminosugar moiety, daunosamine, of daunomycin (or adriamycin) is an essential part of the antibiotic for binding to DNA to take place (*8*). The absence of this aminosugar group in the nogalamycin molecule might explain the lack of fluorescent banding when chromosomes were stained with this anthracycline antibiotic. Nogalamycin and daunomycin have been previously used to induce chromosome banding in vitro (*10*). However, the type of bands obtained with this method could not be positively identified.

There are several aspects of the D-banding technique which are of interest:

1) This procedure clearly differentiates fluorescent patterns on three specific chromosome regions; this allows new research on the specialized structural and functional properties of these regions, and identification of the similar regions that may exist in other species as well.

2) The orange-red fluorescent bands produced by daunomycin and adriamycin do not appear as bright as those produced by the Q-banding technique. However, the fading of fluorescence and the disruptive effect of ultraviolet light on chromosomes is less pronounced as compared with the Q-banding technique. D-bands can still be observed several days after staining. The use of this antibiotic as fluorochromes for chromosome binding is not as sensitive to variations in ionic strength as has been observed in the use of quinacrine (*5*). Thus, it may be more convenient to use the D-band technique for routine chromosome analysis.

3) Anthracycline antibiotics have been shown to have some base specificity in their interaction with DNA (*11*). This class of compounds could provide information on the mechanism of chromosome bands produced by the fluorochrome. Preliminary data suggest that the daunomycin binding patterns may be due to differential fluorescent quenching by DNA regions with specific base sequences (*12*).

The anthracycline antibiotics used in this study are anticanceral agents and are shown to fluoresce brightly in nuclei and meiotic chromosomes of hamster cells shortly after in vivo injection (*13*). Further investigations can be initiated to observe both the biological and the pharmacokinetic effect of the neoplastic cells at a fluorometric level.

C. C. LIN
J. H. VAN DE SANDE
*Divisions of Pediatrics* and *Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta, T2N 1N4, Canada*

### References and Notes

1. T. Caspersson, L. Zech, E. J. Modest, G. E. Foley, U. Wagh, E. Simonsson, *Exp. Cell Res.* **58**, 128 (1969); T. Caspersson, L. Zech, C. Johansson, *ibid.* **62**, 490 (19 ➡ I. Hilwig and A. Gropp, *ibid.* **75**, 122 (1972); M. Bobrow and K. Madan, *Cytogenet. Cell Genet.* **12**, 145 (1973).
2. L. S. Herman, *J. Mol. Biol.* **3**, 18 (1961).
3. R. K. Tubbs, W. E. Ditmars, O. Van Winkle, *ibid.* **9**, 545 (1964); L. M. Chan and J. A. McCarter, *Biochim. Biophys. Acta* **204**, 252 (1970); U. Pachmann and R. Rigler, *Exp. Cell Res.* **72**, 602 (1972).
4. B. Weisblum and P. L. de Haseth, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 629 (1972); E. J. Modest and S. K. Sengupta, in *Chromosome Identification—Techniques and Applications in Biology and Medicine*, T. Caspersson and L. Zech, Eds. (Academic Press, New York, 1973), p. 327; B. Weisblum, *Nature (Lond.)* **246**, 150 (1973).
5. C. C. Lin, H. van de Sande, W. K. Smink, D. R. Newton, *Can. J. Genet. Cytol.* **17**, 81 (1975).
6. D. E. Comings, B. W. Kovacs, E. Avelino, D. C. Harris, *Chromosoma* **50**, 111 (1975).
➡ M. Waring, *J. Mol. Biol.* **54**, 247 (1970); W. J. Ingram, W. Fuller, L. D. Hamilton, *Nat. New Biol.* **235**, 17 (1972).
8. E. Calendi, A. D. Marco, M. Reggiani, B. M. Scarpinato, L. Valentini, *Biochim. Biophys. Acta* **103**, 25 (1965).
9. D. C. Ward, E. Reich, I. H. Goldberg, *Science* **149**, 1259 (1965); W. Kersten, H. Kersten, E. Szybalski, *Biochemistry* **5**, 236 (1966).
10. T. C. Hsu, S. Pathak, D. A. Shafer, *Exp. Cell Res.* **79**, 484 (1973).
11. B. K. Bhuyan and C. C. Smith, *Proc. Natl. Acad. Sci. U.S.A.* **54**, 566 (1965).
12. J. H. van de Sande, C. C. Lin, F. Johnson, in preparation.
13. M. J. Egorin, R. C. Hildebrand, E. F. Cimino, N. R. Bachur, *Cancer Res.* **34**, 2243 (1974); C. C. Lin and J. H. van de Sande, in preparation.
14. This work was supported by Medical Research Council of Canada grants MA-4655 and MA-4885.

27 June 1975

## Antibodies That Impair Insulin Receptor Binding in an Unusual Diabetic Syndrome with Severe Insulin Resistance

Abstract. *Six patients with a unique form of diabetes associated with extreme insulin resistance have markedly reduced insulin binding to specific receptors on their circulating monocytes. When normal insulin receptors were exposed to serum or immunoglobulin fractions from three of these patients in vitro the specific binding defect was reproduced.*

We have studied six patients, all nonobese females, with a unique diabetic syndrome associated with severe insulin resistance and the skin condition acanthosis nigricans (*1*). These patients are unique in that their plasma insulin concentrations, both basal and stimulated by glucose, are increased 10- to 100-fold, and their responses to injected insulin are markedly blunted, with some patients receiving 1000 times the usual dose of insulin to control blood glucose. Furthermore, the specific binding of [$^{125}$I]insulin to insulin receptors on their circulating monocytes is only 5 to 30 percent of normal. In this report, we demonstrate that a serum factor (most probably an antibody) from three of these patients alters the insulin receptor and im-

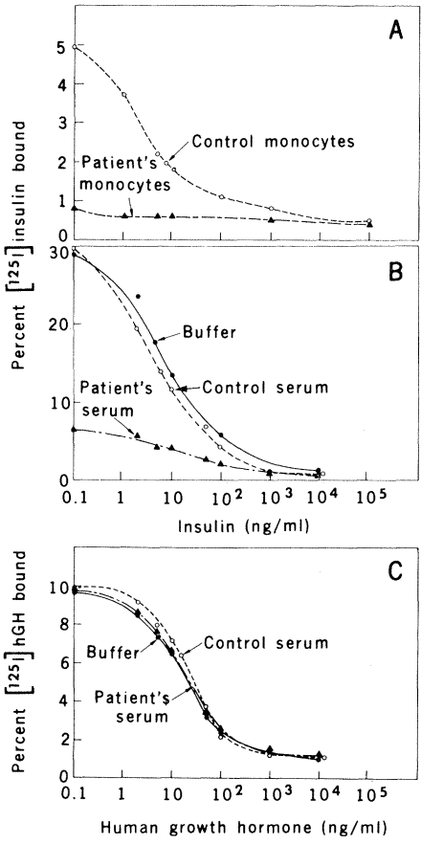



Fig. 1. (A) Binding of [$^{125}$I]insulin to circulating monocytes from a normal control and patient B with the syndrome severe insulin resistance. Mononuclear leukocyte preparations were obtained from buffy coats of each patient by passage through a Ficoll-Hypaque gradient (*9*). Cells were washed and resuspended in HEPES buffer (*p*H 8.0) at a concentration of $50 \times 10^6$ cells per milliliter; $20 \times 10^6$ cells were incubated in 0.5 ml of buffer containing 100 pg of [$^{125}$I]insulin (specific activity, 100 to 200 $\mu$c/$\mu$g) and increasing amounts of unlabeled insulin to give the final concentrations indicated in the figure. After 3 hours at 22°C, duplicate 200-$\mu$l samples were sedimented through 100 $\mu$l of cold buffer in a Beckman Microfuge. Supernatant was aspirated and the [$^{125}$I]insulin bound to the pellet was counted. The number of monocytes, which constitute the major insulin binding cell in this preparation, was determined by ingestion of latex beads (*3*). Binding was expressed as the percentage of [$^{125}$I]insulin bound per $10^7$ monocytes. (B) Effect of serum preincubation on insulin binding to cultured lymphocytes. Human lymphoblastoid cells (IM-9) maintained in continuous culture (*2*) were grown to stationary phase in Eagle's minimum essential medium with 10 percent fetal calf serum. The cells were sedimented and resuspended at a concentration of $15 \times 10^6$ cells per milliliter in HEPES buffer (*p*H 7.6). Samples of cells (0.2 ml) were incubated with 0.2-ml samples of normal serum (control), serum from patient B, or buffer for 60 minutes at 4°C. Cells were then washed three times, once through 1.0 ml of cold fetal calf serum and twice in 1.0 ml of cold buffer. The cells were then resuspended in 0.5 ml of buffer at final concentration of $4.8 \times 10^6$ cells per milliliter and incubated with [$^{125}$I]insulin (100 pg/ml) in the presence of increasing amounts of unlabeled insulin for 90 minutes at 15°C. The percent of [$^{125}$I]insulin bound to duplicate 200-$\mu$l samples was determined as above. (C) Effect of serum preincubation on growth hormone binding to cultured lymphocytes. The IM-9 lymphocytes were preincubated and washed as above; $6 \times 10^6$ cells in 0.5 ml of HEPES buffer (*p*H 7.6) were then incubated with 100 pg/ml of human growth hormone (hGH) labeled with $^{125}$I in the presence of increasing amounts of unlabeled hormone. After 90 minutes at 30°C binding was determined as above.