Pseudomosaicism in Acatalasemic Red Cells Visualized by Fluorescent Antibody Technique

Evidence has been obtained that residual catalase activity in blood of individuals homozygous for acatalasemia is due to the presence of an unstable enzyme variant[1,2]. By means of a modification of the staining technique of KLEIHAUER and BETKE[3], it could be demonstrated that the distribution of this residual activity is uneven, inasmuch as a small number of red cells (about 1%) apparently has normal catalase activity. Fractionation of erythrocytes in a 20% albumin solution has revealed that those cells showing apparently normal catalase activity are mainly found in the least dense fraction, i.e. among the young red cells. However, this technique does not permit a quantitation of the findings because there is a threshold phenomenon. Therefore, it was felt desirable to corroborate this observation by means of the fluorescent antibody technique. This method has previously been used for studying the distribution of other red cell antigens such as HbF[4]. Although only an antiserum against normal human red cell catalase was available, this study seemed feasible because of the previous finding that there apparently is complete antigenic identity between the normal enzyme and its unstable variant[1,5,6].

Methods. For immunization human erythrocyte catalase isolated by the method of HERBERT and PINSENT was used[7]. The preparation obtained had a Kat f value of 60,000. Rabbits were immunized by five 1 ml intradermal injections of 5 mg catalase in Freund's complete adjuvant, given in weekly intervals. Before fluorochrome conjugation, the sensitivity and specificity of the anti-catalase sera were ascertained by the capillary precipitin ring method and OUCHTERLONY's double diffusion test. The technique used for fluorochrome conjugation has been described in detail elsewhere[8,9].

Fractionation of red cells. About 12 ml of ACD-blood was centrifuged at 2800 *g* for 1 h at 4 °C. Serum was removed and buffered saline added to the sedimented cells. After mixing they were centrifuged at 1000 *g* for 15 min. Buffy coat was removed, buffered saline added to the sedimented cells and the tube centrifuged at 2800 *g* for 1 h. After removal of the supernatant the sediment was suspended in twice the volume of 20% human albumin solution. This suspension was centrifuged at 2800 *g* for 2 h at 4 °C. Then successive layers were removed from the tube according to the technique of SASS et al.[10]. The red cell fractions were washed 4 times with buffered saline and suspended in an equal volume of the original serum. From each fraction thin smears were prepared, dried at room temperature and fixed with acetone for 5 min. The fluorochrome conjugate was applied to the smears as described previously[8]. The percentage of catalase positive cells was obtained by counting the cells exerting intense fluorescence out of a total of 1000 cells on photomicrographs. With a similar procedure the HbF staining and counting was performed as described[8].

Results. In order to establish the validity of the fluorescent antibody technique for studying catalase distribution in a red cell population, experiments on artificial mixtures of cells of different catalase level were performed. There is only little fluorescence in mature red cells of acatalasemic individuals, but an intense fluorescence in normal human red cells. Whereas a sharp contrast can be seen in mixtures of acatalasemic cells and those of heterozygotes, there is only little difference in

[1] S. MATSUBARA, H. SUTER and H. AEBI, Humangenetik *4*, 29 (1967).
[2] H. AEBI, E. BOSSI, M. CANTZ, S. MATSUBARA and H. SUTER, in *Hereditary Disorders of Erythrocyte Metabolism* (Ed. E. BEUTLER; Grune and Stratton, New York and London 1968), p. 41.
[3] H. AEBI and M. CANTZ, Humangenetik *3*, 50 (1966).
[4] T. HOSOI, Acta haemat. jap. *31*, 138 (1968).
[5] H. AEBI, M. BAGGIOLINI, B. DEWALD, E. LAUBER, H. SUTER, A. MICHELI and J. FREI, Enzymol. biol. clin. *4*, 121 (1964).
[6] S. MATSUBARA, H. SUTER and H. AEBI, Experientia *22*, 428 (1966).
[7] D. HERBERT and J. PINSENT, Biochem. J. *43*, 203 (1948).
[8] T. HOSOI, Expl Cell Res. *37*, 680 (1965).
[9] T. HOSOI, S. YAHARA, H. B. HAMILTON, N. FUJIKI, T. SASAKI and Y. ISHIHARA, Blood, submitted for publication.
[10] M. D. SASS, C. J. CARUSO and D. J. O'CONNELL, Clinica chim. Acta *11*, 334 (1965).

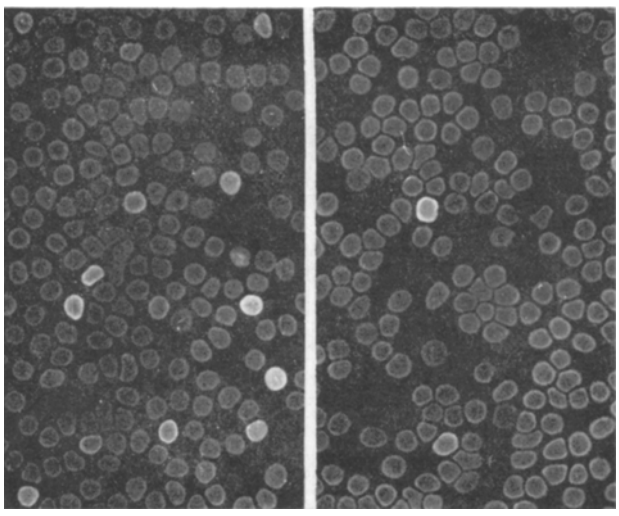

Fig. 1. Blood smears treated with fluorescent anti-human erythrocyte catalase. Blood of Paul G., homozygous for acatalasia. Left: top fraction containing fluorescent (= catalase positive) cells in high proportion (6.5%), the intensity however varying considerably. Right: bottom fraction; fluorescent cells are seen only exceptionally (0.3%).

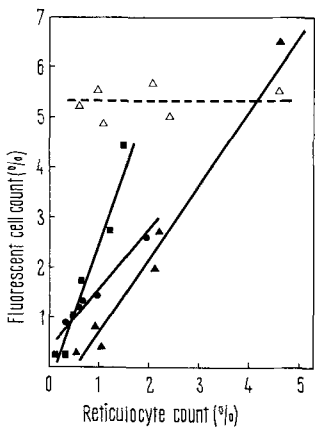

Fig. 2. Correlation between the number of fluorescent (= catalase positive) cells and the reticulocyte count in erythrocyte fractions of varying density. Analysis of one homozygote each of the 3 Swiss type acatalasia families: ■ Andreas B.; ● Marianne V.; ▲ Paul G. For comparison the result of an analogous experiment performed with anti-Hb F (△, Paul G.) has been included.

fluorescence intensity between normal and heterozygous cells.

In blood of individuals homozygous for acatalasemia a pseudomosaicism can be observed (Figure 1), similar to that seen by applying the elution technique[3]. However, using this procedure, it becomes evident that the catalase positive cells do not represent a uniform entity, but seem to be composed of various stages of intermediate fluorescence intensity. In total blood the number of fluorescent (= catalase positive) cells varies between 1–2%, a figure which is slightly higher than that previously obtained using the BETKE staining technique (0.5–1.0%)[3]. Fractionation experiments have revealed that the number of catalase positive cells, as visualized by fluorescent anticatalase, steadily decreases from the top to the bottom fraction. As shown in Figure 2, in each fraction a correlation between the fluorescent cell count and the number of reticulocytes is found. However, throughout this study the number of catalase positive cells has been found to be slightly higher than the reticulocyte count. Members of all 3 Swiss acatalasemia families (type III) have been investigated[2]. They all led to similar results. This procedure of localizing catalase in single cells was compared with an analogue technique visualizing another red cell constituent. For this purpose HbF was stained with a specific fluorescent antibody. The percentage of the HbF-positive cells remains constant, whereas a steady decrease in the number of fluorescent, catalase positive cells is observed from the top to the bottom fraction.

The uneven distribution of residual catalase activity previously found with the elution method is confirmed with the fluorescent antibody technique. However, the identity of catalase positive cells and reticulocytes cannot be proved. The findings reported are consistent with the hypothesis that the low level of catalase activity ($\sim 1\%$) in blood of homozygotes is due to the synthesis of an unstable enzyme variant. A final proof of this concept will only arise from a structural analysis of the catalase variant[11].

Zusammenfassung. Im Blutausstrich lassen sich Erythrozyten von normalem und solche von stark vermindertem Katalasegehalt durch Verwendung von fluoreszierender Antikatalase unterscheiden. Mit dieser Methode konnte der früher erhobene Befund, wonach bei homozygoten Trägern des Enzymdefektes Akatalasie ein Pseudomosaizismus besteht, bestätigt werden. Bei der Untersuchung von Erythrozytenfraktionen verschiedener Dichte besteht eine Korrelation zwischen der Anzahl Retikulozyten und fluoreszierender Zellen. Dieser Befund passt zur Annahme, dass es sich bei der Katalaserestaktivität im Blut homozygoter Defektträger um eine instabile, jedoch antigenidentische Enzymvariante handelt.

T. HOSOI, H. SUTER,
S. YAHARA and H. AEBI

Medizinisch-chemisches Institut der Universität, 3000 Bern (Switzerland) and Kyoto First Red Cross Hospital, Higashiyama-Ku, Kyoto (Japan), 17 December 1968.

[11] Acknowledgment. This investigation has been made possible by the financial aid of the 'Roche' Studien-Stiftung. The cooperation of Drs. J. ROGGO, RIDDES and M. JANN, Altdorf, in obtaining the blood specimens is gratefully acknowledged.

Karyotypic Data for Five Species of Anguid Lizards

MCDOWELL and BOGERT[1] defined an infra-order of lizards, the Anguimorpha, with 2 major phyletic branches, the Diploglossa which retained the more usual and presumably more primitive characters, and the Platynota. Diploglossa includes the wide-spread and species-rich Anguidae, considered to be the most primitive family, and 2 other small extant families. Three subfamilies of Anguidae are recognized[1]: Diploglossinae, presumably the most primitive; Gerrhonotinae, whose ancestry is probably derived from a primitive diploglossine; and Anguinae which is considered close to and derived from Gerrhonotinae. The Gerrhonotinae, containing *Gerrhonotus* and *Ophisaurus*, show a great deal of chromosome variation[2]. We know of no previous chromosome data for the neotropical Diploglossinae.

Previous taxonomic studies on American gerrhonotine lizards, excluding *Ophisaurus*, appear to conflict. TIHEN[3] divided the group into 5 genera: *Gerrhonotus* was monotypic (*G. liocephalus*); *Elgaria* included the species *coerulea, multicarinata, cedrosensis, kingi,* and *paucicarinata*, distributed in Mexico and the western and southwestern United States; and *Barisia* was comprised of 9 species (including *monticola*) that occur in Mexico and Central America. STEBBINS[4] re-examined the species that TIHEN assigned to *Elgaria, Barisia,* and *Gerrhonotus*, and recommended the recognition of a single genus *Gerrhonotus* with 2 subgenera: *Gerrhonotus* (including *liocephalus, multicarinatus, cedrosensis, kingi,* and *paucicarinatus*) and subgenus *Barisia* (including *coeruleus* of western North America, *monticolus* of Costa Rica, and 7 other Mexican and Central American species). A recent osteological study by CRILEY[5] failed to confirm either of these alternative classifications. There were few consistent differences among any of the gerrhonotine lizards. We studied 4 species of *Gerrhonotus* and *Diploglossus costatus* to determine if chromosome data would be useful in clarifying the relationships of these anguid lizards.

Mitotic and meiotic chromosome spreads were obtained from direct testis preparations[6] of 2 *G. multicarinatus multicarinatus* from Napa County, California, 4 *G. coeruleus principis* from Humboldt County, California, 2 *G. monticolus* taken near the summit of Volcan Irazu, Costa Rica, and 4 *G. paucicarinatus* from near La Paz, Baja California. *D. costatus* is a live bearing species. A pregnant female of the subspecies *oreistes*, collected near Kenscoff, Haiti, was injected with Colcemid (Ciba, 0.2 ml of 1 mg/ml solution) 24 h before sacrifice. Two sibling embryos were

[1] S. B. MCDOWELL and C. M. BOGERT, Bull. Am. Mus. nat. Hist. *105*, 1 (1964).
[2] R. MATTHEY, *Les Chromosomss des Vertébrés* (Librairie de l'Université, F. Rouge, Lausanne 1949).
[3] J. A. TIHEN, Am. Midl. Nat. *41*, 580 (1949).
[4] R. C. STEBBINS, Am. Mus. Novit. *1883*, 1 (1958).
[5] B. B. CRILEY, Am. Midl. Nat. *80*, 199 (1968).
[6] G. C. GORMAN, L. ATKINS and T. HOLZINGER, Cytogenetics *6*, 286 (1967).