(*7*). Durand and Espitalie (*8*) determined values for this ratio in the Paris Basin to 2100 m. Their data also show an increase in the concentration of isopentane with depth, causing the ratio to be as high as 2. However, Liebovici and Van der Weide (*9*) calculated equilibrium values for this ratio, and their data indicate that the more stable isomer normal pentane should increase with increasing temperature (depth). Figure 1 shows changes in the pentane ratio with depth in three drill holes in the Black Sea (lines 1, 2, and 3) and one in the Paris Basin (line 4). The calculated equilibrium value is also given.

More recently, we analyzed well cuttings from two coastal offshore stratigraphic tests (COST wells 1 and 2) that were drilled to sediment depths of 4800 and 3962 m, respectively. The wells are located east of Mustang and South Padre islands off south Texas. The data show that the ratio of isopentane to normal pentane follows the same trend as in the Black Sea and Paris Basin. It obeys the calculated equilibrium values at temperatures above about 90°C—although the reason for this is probably kinetic rather than thermodynamic. Normal pentane is dominant in the top 25 m. With increasing sediment depth, there is an increase in isopentane due to low-temperature diagenetic reactions. As the temperature exceeds about 90°C (equivalent to 2290 m in COST well 1), there is an increase in normal pentane from the thermal cracking of the organic matter. Research by Thompson (*10*) supports our observations: in three Gulf Coast samples, he found the ratio of isopentane to normal pentane to be 0.7 at 41°C, 1.7 at 66°C, and 0.9 at 193°C.

As previously stated, near-surface normal pentane is believed to be of biological origin. The isopentane, which appears to increase from the surface to a depth equivalent to a temperature of about 90°C, most likely comes from low-temperature carbonium ion or free radical reactions. Such reactions yield branched hydrocarbons as the dominant products (*11*). Isopentane may also originate from the low-temperature alteration of isoprenoid carbon structures containing functional groups that are widespread in nature. Examples are the phytol chain in chlorophyll, terpenoids, steroids, and many plant and animal pigments. The degradation and reduction of even a small portion of these materials would yield the increasing quantities of isopentane observed with depth to 90°C in marine sediments. At higher temperatures, thermal cracking reactions would gradually acquire enough energy to break carbon-carbon bonds in straight chains to give primary free radicals. This would cause an increase in the percentage of normal pentane in the products. Analyses of several hundred natural gases and about 100 crude oils in the United States and Canada show that the ratio of isopentane to normal pentane is usually around 1 (*12*). Three deep Russian oils have an average ratio of 0.4 (*13*).

Table 1. Mole ratio of isopentane to normal pentane in gases of near-surface marine sediments. Numbers in parentheses give number of samples taken.

Area	Mean	Range
Walvis Bay, Southwest Africa (8)	0.58	0.37–0.94
U.S. Gulf Coast (4)	0.18	0.02–0.57
Black Sea (4)	0.13	0.10–0.18
Arabian Sea (2)	0.69	0.55–0.83
Gulf of Maine (1)	0.54	
Shelf off Nantucket Island (1)	0.50	

In summary, at least three types of reactions are involved in the formation of pentanes and, by inference, of other alkane homologs. The normal pentane initially formed originates biologically or chemically at very low temperatures (< 20°C); therefore its concentration is dependent on biological precursors. The relative increase in isopentane with depth up to about 90°C appears to be a universal phenomenon independent of precursors. It results from low temperature (< 90°C) reactions yielding predominately secondary carbon structures. The relative increase in normal pentane at temperatures above 90°C represents free radical cracking reactions that are also basically independent of source material.

JOHN M. HUNT
JEAN K. WHELAN
Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543
ALAIN Y. HUC
Laboratoire Géologie Appliquée, Centre National de la Recherche Scientifique ERA-601, Orléans, France 45046

References and Notes

1. M. Blumer, R. R. L. Guillard, T. Chase, *Mar. Biol.* **8**, 183 (1971).
2. A. C. Chibnall *et al.*, *Biochem. J.* **28**, 2189 (1934).
3. J. M. Hunt, *Petroleum Geochemistry and Geology* (Freeman, San Francisco, 1979).
4. W. D. Johns and A. Shimoyama, *Bull. Am. Assoc. Pet. Geol.* **56**, 2160 (1972).
5. J. M. Hunt, *Init. Rep. Deep Sea Drill. Proj.* **40**, 649 (1978).
6. ——— and J. K. Whelan, *ibid.* **44**, 651 (1978).
7. ———, *ibid.* **42**, 661 (1978).
8. B. Durand and J. Espitalie, in *Advances in Organic Geochemistry*, H. R. Gaertner and H. Wehner, Eds. (Pergamon, Oxford, 1972), pp. 455–458.
9. C. L. Liebovici, B. M. Van der Weide, in *Advances in Organic Geochemistry*, B. Tissot and F. Bienner, Eds. (Editions Technip, Paris, 1974), pp. 367–378.
10. K. F. M. Thompson, *Geochim. Cosmochim. Acta* **43**, 657 (1979).
11. A. K. Galwey, *ibid.* **36**, 1115 (1972).
12. R. R. Thompson and W. B. Creath, *ibid.* **30**, 1137 (1966).
13. A. N. Sachanen, *Chemical Constituents of Petroleum* (Reinhold, New York, 1945).
14. This work was supported by grant OCE 79-19861 and by DOE contract EG-77-S-02-4392. This is Woods Hole contribution 4549.

26 February 1980; revised 2 May 1980

Antibody-Dependent Cell-Mediated Cytotoxicity: Detection by Automated Flow Cytometry with Ultramicro Techniques

Abstract. *Antibody-dependent cell-mediated cytotoxicity can be measured with as few as 1000 leukocytes with an automated flow cytometry technique.*

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a phenomenon of cell-mediated immunity in which antibody-coated target cells are killed by leukocytes bearing Fc receptors in vitro (*1*). Studies in mice have shown ADCC to be an important component in tumor immunity and immunity to parasitic infections (*2*). Clinical studies of the role of ADCC in human disease, however, have been hampered by the need for large quantities of purified mononuclear cells from peripheral blood, an amount that the patient often cannot afford to lose. The reason for large numbers of cells is directly related to limitations of current assay systems. In a common assay for ADCC, antibody-coated ^{51}Cr-labeled target cells are incubated with a 50- to 100-fold excess of mononuclear effector cells; killing is quantified by measuring release of ^{51}Cr (*3*). Since a minimum of 20,000 target cells is usually needed for statistical precision in gamma counting, each single culture requires 1×10^6 to 2×10^6 purified effector cells. To reduce the number of effector cells needed for ADCC assays one can reduce the ratio of effector cells to target cells (E:T) or reduce the number of target cells, or both. In an attempt to achieve these goals we recently developed an assay for ADCC by automated flow cytometry (FCM) in which target cells are distinguished from effector cells on the basis of cellular DNA content; killing is quantified by monitoring the relative loss of target cells. With this method, significant killing could be detected

0036-8075/80/0718-0404$00.50/0

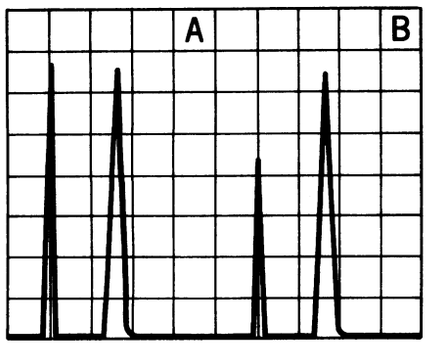

Fig. 1. Representative DNA histograms from ADCC cultures. The horizontal axis represents increasing DNA content; the vertical axis represents relative number of cells. Human effector cells have about 6.0 pg of DNA per nucleus, CRBC about 2.5 pg per nucleus; thus the right-hand peak in (A) and (B) represents effector cells and the left-hand peak represents CRBC target cells. (A) Culture without antibody; (B) culture with antibody to CRBC. Note that in (B) the CRBC peak is greatly diminished, indicating antibody-dependent lysis.

at an *E:T* ratio as low as 0.5:1, a hundredfold improvement in sensitivity (*4*). Since each target cell is counted and measured directly, it seemed possible that one could also use far fewer target cells to measure ADCC.

To test this we measured ADCC with chick red blood cells (CRBC) as target cells; we used 200,000 CRBC in a total volume of 200 μl and 1000 CRBC in a total volume of 4 μl at an *E:T* ratio of 2:1 (*5*). Figure 1 shows representative histograms illustrating the principle of the FCM assay. In Fig. 1B (culture with antibody) the CRBC target peak is diminished in comparison to Fig. 1A (culture without antibody); thus the addition of antibody to cultures leads to target cell lysis. The amount of killing can be quantified by integrating the peaks and calculating the *E:T* ratios of cultures with and without antibody, as described in (*5*). Table 1 shows that in three separate experiments, equivalent values for the ADCC activity of mouse spleen effector were obtained when 200,000 and 1000 target cells were used at the same *E:T* ratio. In other experiments cells from human peripheral blood from three volunteers were purified with a Hypaque-Ficoll gradient (*6*); these cells showed up to 40 percent ADCC with 1000 CRBC targets at a 1:1 ratio (data not shown); thus, as few as 1000 human effector cells can be measured for ADCC activity.

The assay for ADCC by FCM takes only 1 to 2 hours of incubation time compared to 4 hours for the conventional ^{51}Cr assay (*4*). To investigate this discrepancy we examined fixed cells stained with acridine orange from 30-minute ADCC cultures by fluorescence microscopy. The smaller, round nuclei of CRBC were seen to be engulfed by larger phagocytic cells (see cover). To confirm these findings we examined 30-minute cultures by electron microscopy. In Fig. 2 an erythrocyte has been completely engulfed by an effector cell, most likely a macrophage. The conventional theory of ADCC holds that an effector cell bearing an Fc receptor attaches to the antibody-coated target cell, causing membrane changes that lead to lysis of the target and release of ^{51}Cr into the medium (*3*). We and others have shown that the most efficient mediator of ADCC is the monocyte (*1*, *2*); thus our electron microscopy results as well as previously reported time-lapse studies of the interactions of macrophages with antibody-coated red cells (*7*) suggest that contact between target and effector cells is followed by phagocytosis of either a portion of or the entire red cell target within 1 hour. Very little ^{51}Cr would be released into the medium during this interaction, since it would be trapped within the phagocytic effector cell; ^{51}Cr would then be released slowly over a longer period (*8*). With the FCM technique, the cells are lysed with citrate and detergent and bare nuclei are stained for DNA and measured. Thus, while the conventional assay cannot detect release of ^{51}Cr until it leaks out of the effector cell, the FCM technique can readily monitor the destruction and lysis of target cell nuclei by phagocytosis.

Table 1. Assay for ADCC by FCM. Target cells were either 200,000 CRBC in a total volume of 200 μl or 1000 CRBC in 4 μl. Mouse spleen effector cells were added to a final *E:T* ratio of 2:1. After 1½ to 2½ hours of incubation at 37°C, cultures were prepared for FCM analysis (*5*). All cultures were made in duplicate.

Experiment	Percent of killing with	
	200,000 target cells	1000 target cells
1	11 ± 12	19 ± 12
2	37 ± 4	31 ± 8
3	45 ± 5	48 ± 4

The results of our study have useful implications for clinical and experimental immunology. The ability to measure ADCC with only a few thousand effector cells means that studies can be performed on almost any individual without extra venipuncture. The stain solution lyses cell membranes and specifically stains only the nucleus (*9*). Thus whole human blood can be used directly for ADCC without prior Hypaque-Ficoll purification since the nonnucleated red cells would not be detected. We found that whole blood at an *E:T* ratio of 3:1 with 1000 target cells showed 20 to 25 percent ADCC in two separate experiments (data not shown), which is roughly

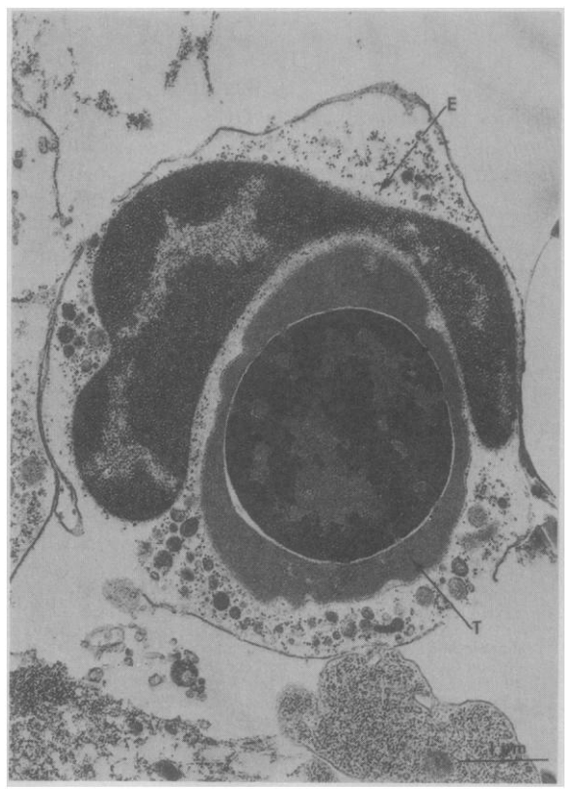

Fig. 2. The ADCC cultures were established (*5*) with 200,000 target and 400,000 effector cells. After incubation at 37°C for 30 minutes, cultures were fixed with glutaraldehyde, dehydrated in graded alcohols, and embedded in plastic. Thin sections were stained with uranyl acetate and examined in an RCA 4A electron microscope; *T*, CRBC target cell; *E*, mouse spleen effector cell. Scale bar, 1 μm.

equivalent to the ADCC of purified human blood at the same ratio. This suggests that only a few microliters of whole blood are needed to measure the ADCC, an amount easily obtained from blood drawn for other diagnostic tests in clinical settings. For animal studies, since only a few microliters of blood are needed for testing, one can serially bleed the same small animal without having to kill it to obtain cells.

Recent studies have shown that up to 1×10^6 mononuclear cells can be extracted from human tumor biopsy samples (*10*); with conventional techniques, functional studies of these cells would be impossible. With the FCM assay, functional studies of extremely small cell subpopulations obtained by cell sorting or gradient density techniques can be envisioned. Circulating immune complexes are found in several disease states such as cancer and parasitic infections (*11*). Since ADCC is mediated by a subpopulation of Fc receptor-bearing cells (*1*), it is extremely sensitive to inhibition by immune complexes. We have shown that the ADCC assay by FCM is more sensitive than conventional techniques for detecting immune complexes (*12*); when the number of target cells is reduced to 1000, the amount of serum needed for immune complex detection becomes minimal. These potential applications of ADCC assay by FCM demonstrate the utility and practicality of flow cytometry in clinical immunopathology in terms of prognosis and diagnosis.

A. M. ATTALLAH
T. J. YEATMAN
P. D. NOGUCHI
J. B. JOHNSON

Division of Pathology, Bureau of Biologics, 8800 Rockville Pike, Bethesda, Maryland 20205

References and Notes

1. P. Perlman and G. Holm, *Adv. Immunol.* **11**, 117 (1969); T. Kivithavongs, W. C. Hottman, J. B. Dossector, *J. Immunol.* **113**, 1178 (1974).
2. P. Hershey, *Nature (London) New Biol.* **224**, 22 (1973); S. E. Order, V. Donahue, R. Knapp, *Cancer* **32**, 573 (1973); A. M. Attallah *et al.*, in preparation.
3. B. R. Bloom and J. R. David, *In Vitro Methods in Cell-Mediated and Tumor Immunity* (Academic Press, New York, 1976).
4. A. M. Attallah, P. D. Noguchi, T. Folks, C. T. Noguchi, *Experientia* **36**, 124 (1980).
5. Procedures have been described in detail in (*4*). Briefly, the CRBC, with and without antibody to CRBC, were mixed with varying amounts of effector cells to give the desired *E:T* ratio. With 200,000 target cells, the total culture volume was 200 μl; with 1000 target cells, it was 4 μl. At the end of 1 to 2½ hours of incubation at 37°C in a humidified atmosphere, the cultures were harvested by aspirating the contents of a culture into 1 ml of dye solution containing 50 μg of propidium iodide (Calbiochem), 0.1 percent trisodium citrate, 0.01*M* NaCl, and 0.1 percent Nonidet P-40 (Bethesda Research Laboratories). After equilibrating in the dye for at least 15 minutes at 4°C, the samples were analyzed by flow cytometry on an Ortho Cytofluorograf, model FC-200. In this instrument cells are individually illuminated by an argon ion laser (488 nm) at a rate of about 500 cell/sec; as each cell passes the laser beam it emits red fluorescent light in proportion to its nuclear DNA content [P. Noguchi and W. Browne, *J. Histochem. Cytochem.* **26**, 761 (1978)]. The signal generated by each cell is converted to a digital value between 1 and 511 by an analog-to-digital converter and stored in an Ortho 2102 multichannel analyzer. The resulting DNA histogram showed two distinct peaks corresponding to CRBC and effector cells (*4*). The number of each cells in each peak was obtained by integration and the *E:T* ratio for each sample calculated from those values. Percent of killing due to ADCC was calculated as $100 \times [1 - (E:T \text{ control})/(E:T \text{ antibody})]$.
6. A. Boyum, *Scand. J. Clin. Lab. Invest. Suppl. 97* **21**, 1 (1968).
7. W. F. Rosse, A. Boisfleury, M. Bessis, *Blood Cells* **1**, 345 (1975).
8. W. A. Walker and A. Demus, *J. Immunol.* **114**, 765 (1975); R. J. Kurlander, W. F. Rosse, G. L. Logue, *J. Clin. Invest.* **61**, 1309 (1978).
9. A. Krishan, *J. Cell Biol.* **66**, 188 (1975); L. L. Vindelov, *Virchows Arch. B* **24**, 227 (1977).
10. J.-L. Svennevig, M. Lovik, H. Svaar, *Int. J. Cancer* **23**, 626 (1979).
11. H. O. Sjogren, I. Hellstrom, S. C. Bansal, G. Warner, K. E. Hellstrom, *ibid.* **9**, 274 (1972); A. M. Attallah *et al.*, *J. Immunol.* **122**, 1413 (1979); C. H. June, C. E. Contreras, L. H. Perrin, P. H. Lambert, P. A. Miescher, *ibid.*, p. 2154.
12. A. M. Attallah *et al.*, in preparation.

9 August 1979; revised 17 October 1979

Carboxyhemoglobin Levels in an Unstable Hemoglobin Disorder (Hb Zürich): Effect on Phenotypic Expression

Abstract. *The affinity of Hb Zürich for carbon monoxide is approximately 65 times that of normal hemoglobin. The carboxyhemoglobin content in serum from individuals with Hb Zürich ranged from 3.9 to 6.7 percent in nine nonsmokers and from 9.8 to 19.7 percent in six smokers. Rates of hemolysis and hemoglobin denaturation were less in smokers than in nonsmokers, effects that may be secondary to the stabilization of Hb Zürich by carbon monoxide.*

Unstable hemoglobins constitute approximately one-fourth of the 350 human hemoglobin variants. The hallmark of these disorders is the propensity of the abnormal hemoglobin to denature and precipitate when intact red blood cells or hemolyzates are exposed to heat, redox dyes, or isopropanol (*1*). The first of the unstable hemoglobins to be analyzed structurally, Hb Zürich (HbZ), is a mutant in which the histidine distal to the heme at position 63 in the β chain is replaced by arginine. Since the original description of the hemoglobinopathy (*2*) two additional and unrelated families have been described in Maryland (*3*). Affected individuals are asymptomatic, with normal blood findings except for mild reticulocytosis. Clinical recognition of the disorder most often depends on the occurrence of anemia and jaundice during infections or following the administration of sulfonamides.

A review of clinical and blood findings in 15 individuals with HbZ, observed by us for 20 years, showed variation in the phenotypic expression of the disorder. One subject did not become anemic after exposure to sulfonamides (*3*); serum concentrations of haptoglobin (an α_2-globulin that binds free hemoglobin) and of hemopexin (a β-globulin that binds free heme) ranged from zero to normal (*4*); and thermally induced denaturation of HbZ in intact red cells was slightly increased in some and markedly increased in others (*4*). These observations led to further investigations to identify factors that may alter the stability of the abnormal hemoglobin and, in turn, its clinical presentation.

The rate of heat denaturation of normal hemoglobin (HbA) and some of the unstable hemoglobins is decreased by the addition of carbon monoxide (CO), a ligand that stabilizes the linkage between heme and globin (*1*, *5*). Because the affinity of HbZ for CO is approximately 65 times that of HbA, an indication that most of the CO is complexed with the abnormal hemoglobin (*6*), excessive exposure to CO of subjects with HbZ may be a factor in modifying the phenotypic expression of the hemoglobinopathy.

The amount of CO bound to hemoglobin in packed erythrocytes from persons with and without HbZ was determined by infrared spectroscopy (*7*, *8*). Normal hemoglobin has one infrared absorption maximum for bound CO at 1951 cm^{-1}. In contrast, purified HbZ has two absorption maxima, one at 1951 cm^{-1} and another, which arises from the abnormal β subunit, at 1958 cm^{-1}. The degree of saturation by CO in vivo was measured as the ratio of integrated CO stretch band intensities at 1951 and 1958 cm^{-1} initially and after saturation with CO.

Percentages of carboxyhemoglobin were measured in 15 asymptomatic HbZ subjects from two unrelated families, together with simultaneous determinations of blood counts, serum concentrations of haptoglobin and hemopexin, and the effects of heat and isopropanol on rates of precipitation of the abnormal hemoglobin in intact red cells and hemolyzates (*9*). Carboxyhemoglobin levels are markedly increased in HbZ individuals who smoke and moderately elevated in those who do not smoke; hematocrit values are higher in the smokers than in the

0036-8075/80/0718-0406$00.50/0