marrow-derived) cells may be more capable of stimulating (*10*) and may be lost differentially remains to be determined. The decrease in the counts per minute of precultured cells when stimulated with PHA suggests some alteration of T (thymus-derived) cells during preculturing.

These findings may lead to a better understanding of the mechanisms of allogeneic cell recognition and stimulation. The common concept of lymphocyte responsiveness being a more active process than the passive stimulation function could prove incorrect.

Loss of immunogenicity upon culturing is important for organ transplantation, as already suggested by Summerlin (*2*). If the loss of immunogenicity of cultured skin is attributed to an effect on passenger lymphocytes, our findings could account for the transplantability of cultured skin. The extent to which this phenomenon can be utilized in the transplantation of bone marrow and other organs is still unknown.

In summary, a basic function of lymphocytes, namely their ability to stimulate allogeneic lymphocytes, has been shown to be independent of another basic function—their ability to respond to allogeneic lymphocytes and to PHA mitogen.

GERHARD OPELZ
PAUL I. TERASAKI
Department of Surgery, School of Medicine, University of California, Los Angeles 90024

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Liquid Chromatographic Assay of Warfarin: Similarity of Warfarin Half-Lives in Human Subjects

Abstract. *A high pressure liquid chromatographic assay was developed to measure warfarin concentrations in biological fluids. Twelve healthy, unrelated volunteers received a single oral dose of warfarin (0.75 mg per kilogram of body weight). The mean plasma warfarin half-life was 36.3 ± 3.5 hours by liquid chromatography but 55.9 ± 8.4 hours by a currently used fluorimetric assay that fails to separate warfarin from its metabolites. Interindividual variation was greater and each half-life longer by the fluorimetric than by the chromatographic procedure. Warfarin shows less interindividual variation than that observed for other drugs primarily metabolized by hepatic microsomal mixed function oxidases. Advantages of specificity, rapidity, sensitivity, accuracy, and simplicity recommend liquid chromatography in the development of other drug assays.*

The oral anticoagulant warfarin is commonly used therapeutically in thrombophlebitis, pulmonary embolism, and myocardial infarction; its measurement in human blood facilitated many investigations on drug interactions and induction of hepatic microsomal mixed function oxidases (*1*). The genetic mechanism for the development of resistance to its use as a rodenticide has been reported (*2*), as have two human pedigrees with resistance to the hypoprothrombinopenic effects of warfarin (*3*). Elucidation of these phenomena depended on measurement of warfarin in biological fluid by a spectrophotometric (*4*) or fluorimetric assay (*5*). More recently, Lewis *et al.* (*6*) introduced a method for warfarin determinations based on thin-layer chromatography which separated warfarin from its metabolites. Lewis *et al.* (*6*) also showed that, when assayed for warfarin, blood specimens drawn serially after a single oral dose of warfarin in human subjects gave different clearance rates by the spectrophotometric (*4*) and the fluorimetric (*5*) methods. However, Welling *et al.* (*7*), using a smaller oral dose of warfarin, failed to confirm the observations of Lewis *et al.* (*6*). Lewis (*8*) refuted Welling *et al.* (*7*), and Wagner (*9*) replied. Hewick and McEwen (*10*) confirmed Lewis's work and extended his observations by reporting different plasma half-lives and anticoagulant efficacies for the enantiomers of warfarin in man.

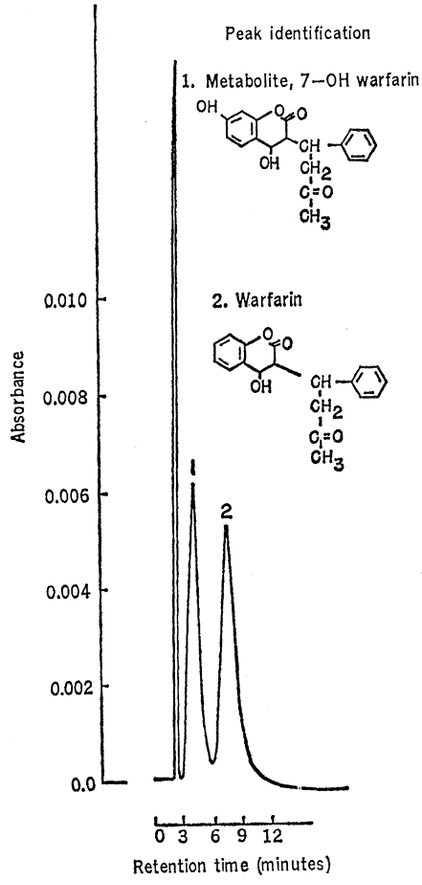

Fig. 1. Separation of warfarin and 7-hydroxywarfarin by high pressure liquid chromatography.

Increasing interest in warfarin pharmacokinetics and in measuring concentrations of other drugs prompts us to describe a simple assay of warfarin by high pressure liquid chromatography (LC) and to suggest the suitability of LC for quantitative estimation of other drugs. Advantages of LC over gas-liquid chromatography include elimination of an occasionally difficult derivatization step and also of volatilization which may fragment certain thermally unstable compounds.

The DuPont model 830 high pressure liquid chromatograph was used. A column (1 m by 2.1 mm) was packed with octadecylsilane (Permaphase). The mobile phase consisted of 10 percent dioxane in 90 percent H_2O at *p*H 4. The flow rate was 0.75 ml/min. The

column input pressure was 1000 lb/in.^{2} (1 lb/in.^{2} = 0.068 atm); the temperature 25°C; the injection volume 6 μl; and the sensitivity 0.02 absorbance unit full scale. The detector was an ultraviolet photometer at a fixed wavelength of 254 nm. The chart speed was 10 minutes per inch (1 inch = 2.54 cm). Separation of warfarin (K and K Labs) from authentic 7-hydroxywarfarin (courtesy of R. Lewis), both added to human plasma, is shown in Fig. 1. A reversed phase system at *p*H 4 was used to separate warfarin from its metabolite 7-hydroxywarfarin. The low *p*H suppressed ionization of the warfarin and enhanced its solubility in the nonpolar stationary phase, thus permitting the polar mobile phase to elute the metabolite before warfarin. Before addition of these authentic compounds, whose identities were verified by thin-layer chromatography and determination of the melting point, the plasma showed neither peak on LC. To assay warfarin, extraction from plasma into ethylene dichloride was performed as previously described (*4, 5*). The ethylene dichloride phase was then dried under a stream of nitrogen. The residue was dissolved in 25 μl of *p*-dioxane; 6 μl were then injected directly into the liquid chromatograph. A standard curve for the LC assay of warfarin added to normal human plasma is linear up to 10 μg/ml. The coefficient of variation by this method was 2.0 percent.

Twelve healthy, unrelated adult male volunteers (aged 21 to 29), who for 2 weeks preceding the experiment received no medications, took sodium warfarin (5 and 10 mg tablets from Endo Laboratories) in a single oral dose of 0.75 mg/kg. At 24, 36, 48, 60, and 72 hours after warfarin administration, blood specimens of 14 ml each were drawn by venipuncture into tubes containing 0.1 ml of 40 percent sodium citrate. Plasma concentrations of warfarin were determined on each specimen both by the fluorimetric method (*5*), which gave a mean warfarin half-life ± standard deviation (S.D.) of 55.9 ± 8.4 hours, and by LC, which showed a mean warfarin half-life ± S.D. of 36.3 ± 3.5 hours (Fig. 2). The range in half-lives estimated by LC was from 30.6 to 40.0 hours or 33 percent. For each specimen the value obtained by LC was less than that obtained by fluorimetry, presumably because of inclusion of fluorescent metabolites with warfarin in the fluorimetric procedure. However, warfarin metabolites were undetected under the LC conditions described above. Possibly by altering sensitivity, extraction methods, or other conditions warfarin metabolites could be identified by LC. The LC assay measured both isomeric forms of warfarin and both free and bound portions of the drug in plasma. In the LC assays, the extrapolated *y*-intercept was similar for most subjects, with the exception of B.B., G.W., and T.B. (Fig. 2), indicating very small interindividual variations in apparent volumes of distribution of warfarin. By contrast, in patients taking different doses of warfarin for at least 3 months to achieve anticoagulant control, both the plasma half-life and apparent volume of distribution of warfarin showed large interindividual variations (*11*).

In addition to suggesting advantages of high pressure LC in measuring warfarin concentrations in plasma and in its potential applications in separating other drugs from their metabolites, these results reveal surprisingly small interindividual variation in plasma warfarin half-life in healthy, nonmedicated, unrelated human subjects. By contrast, phenylbutazone, antipyrine, bishydroxycoumarin, ethyl biscoumacetate, nortriptyline, and diphenylhydantoin exhibit interindividual variations ranging from threefold to more than 40-fold in plasma half-life in healthy, nonmedicated, unrelated human subjects (*12*).

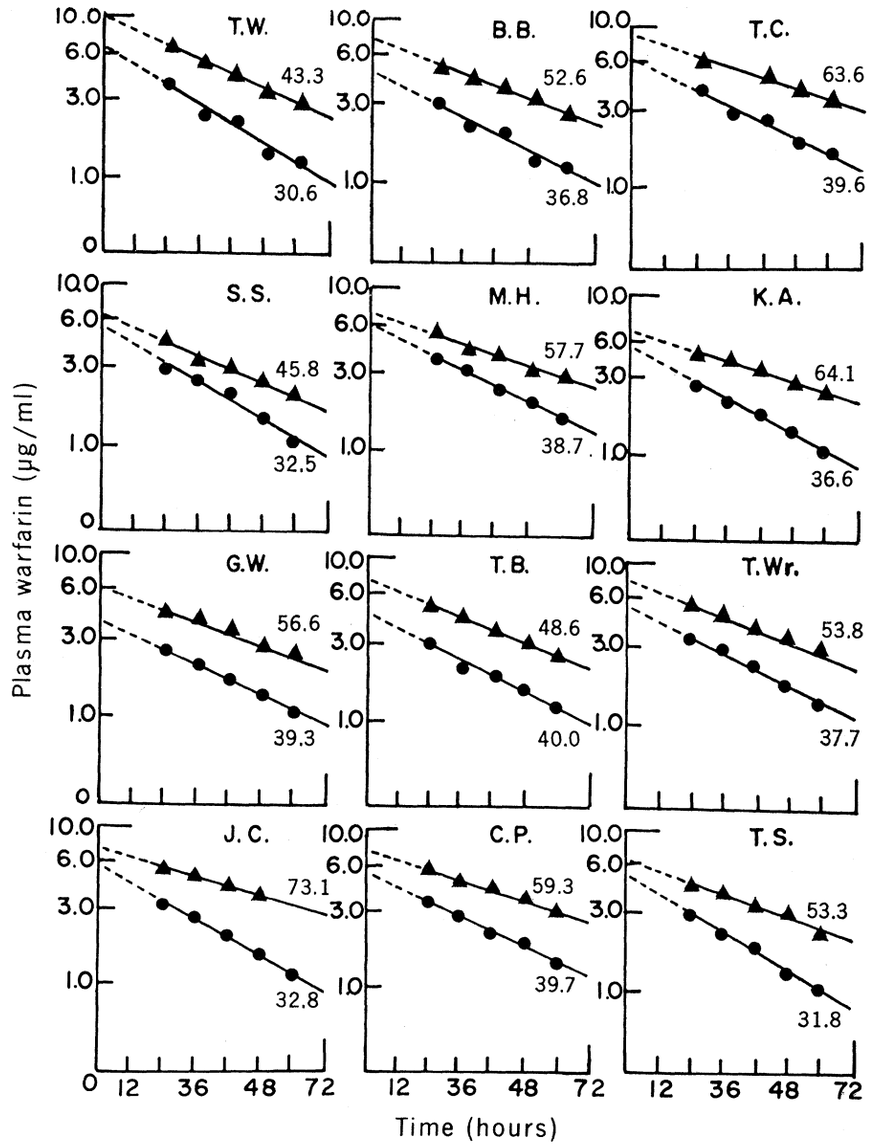

Fig. 2. Decay of warfarin in human plasma after a single dose of warfarin (0.75 mg/kg) to 12 volunteers as measured by the fluorimetric (▲) and liquid chromatography (●) methods. All values for half-life were determined by least squares analysis.

Attributable mainly to genetic factors, these large interindividual variations are responsible for some cases of drug toxicity arising when "usual" doses of these drugs are administered to all subjects without appropriate modification (*12*). For these drugs, the assays employed exclude metabolites (*12*). Although warfarin, like the aforementioned drugs, is metabolized extensively by hepatic mixed function oxidases, warfarin plasma half-lives and apparent volumes of distribution are very similar in unrelated, nonmedicated, healthy subjects. To test whether the 12 subjects with similar warfarin half-lives were also homogeneous with respect to the elimination of other drugs, we determined at a later time their plasma antipyrine half-lives after a single oral dose of antipyrine (18 mg/kg). Plasma antipyrine half-lives varied more than threefold, ranging from 6.5 to 21.0 hours; the intraindividual correlation between plasma antipyrine and warfarin half-lives was .56, not quite attaining significance ($.10 > P > .05$). The currently widely accepted tenet that in healthy, nonmedicated subjects all drugs metabolized primarily by this hepatic microsomal system exhibit large interindividual variations in rates of elimination should be reexamined.

ELLIOT S. VESELL
CAROL A. SHIVELY
Department of Pharmacology,
Pennsylvania State University
College of Medicine, Hershey 17033

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Heterocytolysis by Macrophages Activated by Bacillus Calmette-Guérin: Lysosome Exocytosis into Tumor Cells

Abstract. *The cytotoxic activity of activated macrophages against tumorigenic target cells appears to be mediated by lysosomal enzymes of activated macrophage origin. Lysosomes of activated macrophages are secreted directly into the cytoplasm of susceptible target cells, which subsequently undergo heterolysis. This reaction can be inhibited by agents which prevent the exocytosis of macrophage lysosomes (hydrocortisone) or which interfere with the action of lysosomal enzymes (trypan blue).*

Mouse peritoneal macrophages, activated in vivo by chronic infection by BCG (bacillus Calmette-Guérin) or toxoplasma, or in vitro by endotoxin, destroy tumorigenic cells by a nonphagocytic mechanism requiring direct contact. This cytotoxic reaction is based on a system of nonimmunologic discrimination at the effector level; in this reaction activated macrophages have little or no cytotoxic activity for nontumorigenic cells and normal macrophages are not cytotoxic (*1*, *2*).

Phase contrast microscopy and lysosomal markers were used to show that lysosomes are transferred from activated macrophages to the cytoplasm of susceptible target cells that subsequently undergo heterolysis; a similar transfer of lysosomal markers was not noted with normal macrophages. It was observed that the cytotoxic effect of activated macrophages is inhibited by hydrocortisone, a membrane stabilizer (*3*) which interferes with the transfer of lysosomal markers to tumorigenic target cells. This suggests that hydrocortisone prevents membrane fusion reactions required for lysosome exocytosis into susceptible target cells. In addition, the cytotoxic effect of activated macrophages can be inhibited with trypan blue, an inhibitor of lysosomal enzyme action (*4*). Activated macrophages readily transfer the trypan blue lysosomal marker to susceptible target cells but the target cells continue to undergo rapid mitosis. These results suggest that the cytotoxic action of activated macrophages can be inhibited either by preventing exocytosis of lysosomes or by directly inactivating lysosomal enzymes secreted into target cells. Therefore, it appears that lysosomal enzymes of activated macrophage origin are the effectors of target cell destruction.

These studies were performed with macrophages activated by chronic infection with BCG or toxoplasma. Macrophages activated by these two infections had identical cytotoxic activity. The cytotoxicity tests were prepared as described (Table 1) unless otherwise indicated.

Figure 1A shows a thick multilayer of tumorigenic 3T12 cells surrounding a central monolayer of BCG-activated macrophages. The destruction of 3T12 cells occurred only where they were in contact with activated macrophages. This is evidence that the cytotoxic mechanism does not involve a soluble cytotoxic mediator (SCM). To further test for a possible SCM elaborated by activated macrophages, we removed culture medium every 3 hours and each time replaced it with fresh medium warmed to 36.5°C. The cytotoxic effect was identical to that of control cultures (where the medium was not changed), suggesting that destruction was independent of an SCM whose effect could be diminished by dilution. To promote the thorough distribution of an SCM, we mixed the culture medium with a sterile Pasteur pipette every 60 minutes during the 72-hour incubation period. This did not interefere with 3T12 cell destruction among activated macrophages nor did it produce inhibition of 3T12 cell growth on the periphery of the cover slip which was free of activated macrophages. It was also possible that an SCM was present but active only in cooperation with macrophages. To test this, we removed the supernatant medium after ..., 48, and 72 hours of incubation from monolayers of activated macrophages or from activated macrophages that had been challenged with 3T12 cells. Both types of activated macrophage-conditioned medium were added to normal macrophages that had been challenged with 3T12 cells 1 hour earlier, and the cultures were evaluated for cytotoxic effect after a 72-hour incubation period. Activated macrophage-conditioned medium did not render normal macrophages cytotoxic. These experiments and those of earlier studies (*1*, *5*) failed to demonstrate SCM activity associated with the activated macrophage cytotoxic reaction.

Nonenzymatic agents that accumulate