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COUMARIN OR WARFARIN TREATMENT OF MICE DOES NOT INCREASE THE MICROBICIDAL OR TUMORICIDAL CAPACITIES OF MACROPHAGES

G. A. FILICE* AND J. S. REMINGTON

From the Division of Allergy, Immunology and Infectious Diseases, Palo Alto Medical Research Foundation, Palo Alto, California 94301, *and the Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, Stanford, California* 94305, *U.S.A.*

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Summary.—Benzopyrones have been shown to affect several functions of macrophages. We examined the effects of two benzopyrones, coumarin and warfarin, on the capacity of mouse macrophages to inhibit microorganisms and tumour target cells. Mice were treated with daily i.v. doses of either drug. Then the mice were challenged with lethal doses of *Toxoplasma gondii* or peritoneal macrophages from these mice were challenged *in vitro* with *T. gondii* or tumour target cells. Survival of coumarin- or warfarin-treated mice challenged with *T. gondii* was similar to that of control mice. Multiplication of *T. gondii* and growth of tumour target cells were similar in preparations of macrophages from coumarin-treated, warfarin-treated, or control mice and were inhibited in preparations of activated macrophages from *Corynebacterium parvum*-treated mice that served as positive controls. Under our experimental conditions, benzopyrones did not activate mouse macrophages.

SEVERAL STUDIES have suggested that benzopyrones or their derivatives perturb the reticuloendothelial system and, in particular, affect certain functions of macrophages. For instance, clearance of colloidal gold was found to be greater in patients taking anticoagulants that are chemically related to coumarin (5-6-benzopyrone) than in control patients (Adlercreutz and Pettersson, 1963). Treatment of rats with two benzopyrones, coumarin and rutin, causes increased migration of macrophages on to glass cover slips and increased spreading of these macrophages (Piller, 1978*a*). These results led us to hypothesize that benzopyrones or their derivatives might activate macrophages so that they would inhibit multiplication of microorganisms or tumour cells.

Most of the studies of the effects of benzopyrones on the reticuloendothelial system have used coumarin alone or in combination with other benzopyrones. Since coumarin is the parent compound of warfarin and other anticoagulants, some of which are frequently used in man, we considered the effects of coumarin and its derivatives on the functional status of mononuclear phagocytes to be of interest and of potential importance. To this end, we examined the effects of coumarin and warfarin on the capacity of mouse peritoneal macrophages to inhibit microorganisms and tumour cells.

MATERIALS AND METHODS

Treatment of mice with coumarin, warfarin, or Corynebacterium parvum.—Six-week-old Swiss Webster female mice (Simonsen Laboratories, Gilroy, California) were used in all experiments. For 4 days, mice received daily i.v.

* Present address: Division of Infectious Diseases, Veterans Administration Medical Center, Minneapolis, Minnesota 55417, U.S.A.

Correspondence to: Jack S. Remington, M.D., Palo Alto Medical Research Foundation, 860 Bryant Street, Palo Alto, California 94301, U.S.A.

injections of coumarin (dissolved in 2% ethanol; Eastman Kodak Company, Rochester, New York), warfarin (kindly provided by Endo Laboratories, Inc., Garden City, New York), saline, or 2% ethanol in saline. Daily doses of coumarin were 25 mg/kg, which is similar to doses shown in previous studies to affect the reticuloendothelial system (Piller, 1977). Daily doses of warfarin were 0·1, 0·2, 0·4, 0·8, 1·6, 6·25, 12·5 and 25 mg/kg for all types of experiments except as noted. A dose of 0·1 mg/kg/day is similar to doses of warfarin used in man for anticoagulation. Activated macrophages were used as a control in some experiments and were obtained from *C. parvum*-injected mice. *C. parvum* (Lot CA 582A, 1·4 mg/mouse; kindly provided by Dr John Whisnant, Wellcome Reagents Ltd, Research Triangle Park, North Carolina) was injected i.p. 7 days before macrophages were harvested for study (Krahenbuhl, Lambert and Remington, 1976).

Infection of mice with T. gondii.—Groups of coumarin-treated, warfarin-treated, or control mice (2–7 mice per group) were infected with either the RH strain or the C56 strain of *Toxoplasma gondii*. Tachyzoites of the RH and C56 strains were obtained as previously described (Krahenbuhl, Ruskin and Remington, 1972). Five thousand tachyzoites of the RH strain or 5×10^5 tachyzoites of the C56 strain were injected into the tail veins of groups of coumarin- or warfarin-treated or control mice 3 h after the first treatment. Mortality in all groups of mice was recorded daily. All mice were examined *post mortem* for macroscopic evidence of haemorrhage.

Preparation of macrophage monolayers.—Macrophages were obtained from mice that were given 1 dose of coumarin, warfarin, or saline on each of the preceding 4 days. Peritoneal exudate cells were harvested in Hanks' balanced salt solution and macrophage monolayers were prepared using Medium M199 with 10% foetal calf serum (M199–FCS) as previously described (McLeod and Remington, 1977*a*). After 2 h of incubation, non-adherent cells were washed off and the remaining cells (macrophages) were challenged with *T. gondii* or tumour cells.

Infection of macrophages with T. gondii.—Tachyzoites of the RH strain were suspended in M199–FCS and used to challenge macrophages in a ratio of 2 tachyzoites per macrophage (Anderson and Remington, 1974; Wilson, Tsai and Remington, 1980). At intervals, slides were fixed and stained and the degree of multiplication was assessed microscopically (Anderson and Remington, 1974; Wilson *et al.*, 1980).

Tumour inhibition assay.—To determine whether macrophages could inhibit the growth of tumour cells, macrophage monolayers were challenged with EL-4 cells in M199–FCS. Thymidine incorporation into trichloroacetic acid (TCA) precipitable material by EL-4 cells was measured as previously described (Krahenbuhl and Remington, 1974). A cytostatic index was defined as $[(N\text{-}E)/N] \times 100$, where N is counts per min (cpm) of the TCA precipitable material from EL-4 cells exposed to normal macrophages and E is cpm of the TCA precipitable material from EL-4 cells exposed to experimental macrophages (Ghaffar *et al.*, 1974).

RESULTS

Effect of coumarin or warfarin treatment on survival of mice challenged with T. gondii

All mice challenged with *T. gondii* died, and the interval between infection and death was the same for coumarin- or warfarin-treated and control mice. Mice lived from 7 to 14 days after injection of the C56 strain of *T. gondii* and from 7 to 8 days after injection of the RH strain of *T. gondii*. Haemorrhage did not appear to obscure any protective effect of coumarin or warfarin, as the proportions of mice with haemorrhage in the drug-treated and control groups were similar. Nineteen per cent of all infected mice had macroscopic evidence of bleeding at necropsy, usually apparent as a small amount of blood in the stomach or intestine. The haemorrhage was not massive in any. The higher doses of warfarin were not associated with an increased incidence of haemorrhage.

To determine if warfarin would protect mice if warfarin injections preceded *T. gondii* challenge by more than 3 h, we began warfarin injections (0·1 mg/kg/day) 27 h before challenge with *T. gondii* in 1 group of mice and continued daily warfarin injections for a total of 5 days. Mortality in these mice was similar to mortality in saline-treated controls.

Effects of macrophages on T. gondii in vitro

Macrophages from *C. parvum*-treated mice markedly inhibited multiplication of *T. gondii*. In contrast, macrophages from mice treated on each of the preceding 4 days with either coumarin, warfarin, or saline did not inhibit multiplication of *T. gondii*. One hour after challenge, the

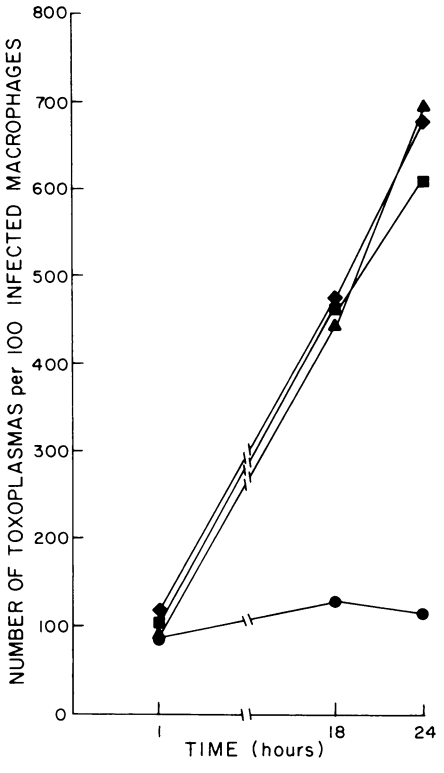

FIG.—Multiplication of Toxoplasmas within macrophages from *C. parvum*- (●), coumarin- (■), or warfarin- (▲) treated or control (◆) mice. A rise in the mean number of Toxoplasmas per 100 infected macrophages reflects intracellular multiplication of the Toxoplasmas.

proportion of macrophages containing recognizable Toxoplasmas was approximately 25% for macrophages from all groups of mice. By 18 and 24 h after challenge, the proportion of macrophages from *C. parvum*-treated mice that contained recognizable Toxoplasmas dropped to 10%, suggesting disintegration of some of the parasites within these cells. At these later times, the proportions of macrophages from the other groups of mice that contained recognizable Toxoplasmas remained the same as at 1 h. At 18 and 24 h after challenge, Toxoplasmas had multiplied extensively within macrophages from coumarin-, warfarin-, and saline-treated mice. The extent of multiplication was similar in macrophages from these three groups of mice (Fig.).

Effects of macrophages on tumour cells

Thymidine incorporation into TCA-precipitable material was significantly less for EL-4 cells incubated with macrophages from *C. parvum*-treated mice than for EL-4 cells incubated with macrophages from control mice (Table). In contrast, thymidine incorporation was similar for EL-4 cells incubated with macrophages from coumarin- or warfarin-treated and control mice.

TABLE.—*Effect of macrophages on tumour target cells*

Source of macrophages	Inhibition of thymidine incorporation (CI)*
C. parvum-treated mice	91†
Coumarin-treated‡ mice	9
Warfarin-treated‡ mice	14

* CI = cytostatic index.

† Incorporation in the presence of macrophages from *C. parvum*-treated mice was significantly less ($P < 0{\cdot}001$, Student's t test) than incorporation in the presence of macrophages from normal mice.

‡ Dose = 25 mg/kg/day.

DISCUSSION

Previously reported evidence that benzopyrones perturb the reticulo-endothelial system led us to hypothesize that coumarin, or its derivative warfarin, might activate macrophages and thereby enable them to inhibit or kill microorganisms or tumour cells. In the *in vivo* mouse model of Toxoplasma infection used in our study and in which activated macrophages contribute in a major way to defence (McLeod and Remington, 1977*b*), coumarin or warfarin treatment did not affect survival of the mice. In our *in vitro* experiments, we found that administration of coumarin or warfarin to mice did not change the abilities of their macrophages to inhibit multiplication of *T. gondii* or of tumour cells. Thus, using these functional assays of activation, we found no evidence that

coumarin or warfarin activates mouse macrophages.

Several lines of evidence from previously reported studies suggest that coumarin alters properties of macrophages from experimental animals. In rats, coumarin increased the initial rate of formation of inflammatory oedema but reduced the total volume of oedema fluid (Piller, 1977). Silica treatment, intended to inactivate macrophages, abrogated the effect of coumarin on the volume of inflammatory oedema *in vivo* (Piller, 1976), and coumarin enhanced catabolism of albumin by macrophages *in vitro* (Bolton and Casley-Smith, 1975). Coumarin treatment, alone or in combination with another benzopyrone, has affected resorption of hyphemas in guinea pigs (Piller, 1978*b*), spontaneous migration of inflammatory cells from rats (Dunn *et al.*, 1977), and phagocytosis of latex particles by mouse peritoneal macrophages (Dunn *et al.*, 1977). In coumarin-treated rats (Piller, 1978*a*) and mice (Koh and Willoughby, 1979), macrophages migrated on to subcutaneous glass coverslips in greater numbers and exhibited greater spreading than macrophages in control rats. Warfarin has also been shown to reduce the total volume of inflammatory oedema in rats (Eichbaum, Slemer and Zyngier, 1979).

The effects of benzopyrones on the reticuloendothelial system in man are less well understood. In one non-randomized, non-blind clinical study (Piller and Clodius, 1976), coumarin and troxorutin (another benzopyrone) were given to a group of patients with lymphoedema of one arm. Resistance to external compression decreased in the arms of treated patients. No data on changes in arm circumference were given. In another study (Edwards and Rickles, 1978), it was found that warfarin treatment decreased skin-test induration and tissue-factor generation while lymphocyte transformation remained unaffected. In another study (Adlercreutz and Pettersson, 1963), clearance of colloidal gold from the circulation in patients receiving phenylpropylhydroxycoumarin or phenyl-indanedione was greater than in controls. To our knowledge, no studies have been done to examine directly the effects of benzopyrones on human mononuclear phagocytes. Our results suggest that it is unlikely that treatment of humans with coumarin or warfarin will activate mononuclear phagocytes.

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