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Differences between Vinblastine and Vincristine in Distribution in the Blood of Rats and Binding by Platelets and Malignant Cells*

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Abstract—*Tritium labeled vinblastine (VLB) and vincristine (VCR) were injected i.p. at a subacutely-toxic dose* (0.25 mg/kg) *into rats (Nb strain). In the first few hours the levels of VLB in the blood were substantially higher than those of VCR; subsequently, however, the levels of blood radioactivity in the case of VLB fell much more rapidly than those of VCR, reaching values well below the latter in* 8–10 hr. *There was a correlation between the different blood labeling profiles of the alkaloids and their binding by platelets—the carriers of a major proportion of the circulating alkaloids.*

In vitro, *platelets, cells isolated from alkaloid sensitive rat lymphomas and cultured L5178Y cells, all took up VLB much more rapidly than VCR; when restored to alkaloid free medium the cells readily released VLB but, in contrast, retained VCR tenaciously. In binding to cells, VLB and VCR have similar equilibrium association constants and are apparently bound to common receptors.*

It is suggested the preferential retention of VCR by cells may be an important factor underlying the greater toxicity and oncolytic potency of VCR.

INTRODUCTION

VINBLASTINE (VLB) and vincristine (VCR), cytotoxic alkaloids isolated from the plant *Catharanthus roseus* [1, 2], are used extensively in the treatment of certain types of cancer [3, 4]. The two compounds are very similar in structure (Fig. 1) and apparently also in their primary biological action. Thus the toxicity of VLB and VCR can in many cases be linked to disturbances in intracellular microtubular structures resulting from the binding of the alkaloids to a common target molecule, tubulin, the protein subunits from which microtubules are assembled [5–9]. In view of their structural and biological similarities it is of considerable interest that in their use as anticancer agents VLB and VCR differ markedly in their spectra of oncolytic activity, the dosages which are tolerated and the symptoms of toxicity they elicit. VLB, for example, is one of the more useful drugs for treating Hodgkin's disease but has only minimal effects against acute leukemia; VCR, on the other hand, is a valuable drug in the treatment of acute leukemia, particularly in children, and is also effective against certain lymphomas and neuroblastomas [3, 4]. VCR is substantially more toxic to the host than VLB (e.g. tolerated dose for adults: VCR, 25 μg/kg; VLB, 150 μg/kg). The main dose limiting factor in the use

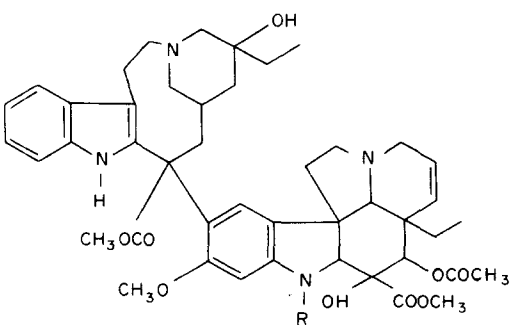

Fig. 1. Vinblastine, R=—CH_3; *vincristine, R*=—CHO.

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of VCR is neurotoxicity which may be manifested as a severe neuropathy; the dose of VLB on the other hand is usually limited by its toxic effects on the hematopoietic system (e.g. bone marrow depression, leukopenia) [3, 4].

The biochemical and biological evidence presently available does not provide an explanation of the marked differences in the toxic and oncolytic properties of VLB and VCR. The differences may presumably arise from a number of variables, including the delivery of the administered alkaloids to the tissues and cells with which they interact, the alkaloid concentrations to which the tissues and cells are exposed, the transport of the alkaloids across the plasma membrane to intracellular receptors (e.g. tubulin) and the affinity of the receptors for the alkaloids. The identification and quantitation of the pharmacological, cytological and biochemical parameters which mediate the effects of the alkaloids may lead to a better understanding of their mechanisms of action and to an explanation of the differences between them. In the present study we have compared the pharmacokinetics of distribution of VLB and VCR in the peripheral blood of rats and the binding and release of the alkaloids by alkaloid sensitive cells. The cell types used have included blood platelets (which previous studies indicate may play a role in the transport of the alkaloids in the body [10, 11]) and cells isolated from a line of rat lymphomas which in their response to treatment with the alkaloids resemble certain human malignancies [12, 13]. Marked differences have been found between the two alkaloids both in the pharmacokinetics of their distribution in the blood and in their binding and release by cells. It is suggested that the differences in the cell binding properties of the alkaloids may account, at least in part, for some of the differences in their cytotoxic and oncolytic properties.

MATERIALS AND METHODS

Alkaloids

VLB sulfate and VCR sulfate were gifts from Eli Lilly & Co., Indianapolis, Ind., U.S.A.

Preparation of tritiated VLB and VCR

Tritiated VLB ([^{3}H]VLB) was prepared from VLB by proton exchange with tritiotrifluoroacetic acid and purified by procedures already described [11]. Tritiated VCR ([^{3}H]VCR) was prepared from VCR by the same method and purified by successive chromatography on columns of alumina and cellulose phosphate [10, 14]. The radiochemical purity of the tritiated alkaloids, based on co-chromatography with carrier compounds and isotopic dilution analysis, was: [^{3}H]VLB, 97.8%; [^{3}H]VCR, 95.0%. Their specific radioactivities were: [^{3}H]VLB, 1.4×10^9 dis/min/mg; [^{3}H]VCR, 1.6×10^9 dis/min/mg. The biological activity of the tritiated alkaloids, e.g. inhibition of cell replication, metaphase arrest in cultures of L5178Y cells, was the same as that of the corresponding non-radioactive compounds.

Rats

The animals used in this study were Nb strain rats bred in the animal colony of the Cancer Research Centre, University of British Columbia [15]. Male rats, wt 225 ± 25 g, were given i.p. injections of the tritiated alkaloids (0.25 mg/kg) dissolved in 1 ml of 0.9% NaCl solution.

Tumors

Lymphomas of the Node lymphoma No. 2 line were maintained by subcutaneous transplantation in the neck region of Nb strain rats. As reported elsewhere [13, 15] this lymphoma grows rapidly to give well defined masses with minimal necrosis and hemorrhage, and even when far advanced responds well to treatment with either VLB or VCR, regressing rapidly until no longer palpable. On a mg/kg basis VCR is 2–3 times more effective than VLB [13].

Blood sampling and fractionation

Blood was sampled from the tail or heart of the rats [10]. Blood plasma, WBC and RBC fractions were obtained by centrifuging citrated blood for 10 min at 4300 ***g*** in polyethylene tubing and separating the fractions by cutting the tubing with a razor blade as previously described [10, 11].

Preparation of platelet rich plasma

Platelet rich plasma (PRP) was prepared from heart blood of lightly etherized rats by the method of Aster and Jandl [16]. The blood (8 ml) was drawn into a 10 ml plastic syringe containing 1.6 ml of 0.15 M citrate (pH 4.6) as anticoagulant. After mixing, the citrated blood was expelled into a plastic centrifuge tube, allowed to stand

at room temperature for 20 min and then centrifuged for 15 min at 275 ***g***. The supernatant and WBC layer were removed with a siliconized pipette as one fraction which was then centrifuged for 5 min at 400 ***g*** to pack down erythrocytes and leukocytes. The supernatant was removed and used as "platelet rich plasma". The platelet count was $1.0 \pm 0.2 \times 10^6/mm^3$; red cell and leukocyte contamination was very low.

Preparation of cell suspensions from rat lymphomas

Rats bearing rapidly growing transplanted Node lymphomas (1.5–2.5 cm dia) were etherized and the tumors excised. Portions of the tumors, free of necrotic and hemorrhagic areas, were minced with scissors in a mixture of Hanks' balanced salt solution (6 vol) and calf serum (4 vol), buffered at pH 6.8 with citrate (0.015 M). The resulting suspension was filtered through stainless steel gauze to remove pieces of tumor tissue giving a suspension of cells which was usually contaminated with a substantial number of platelets. In view of the high affinity of platelets for VLB and VCR and the errors which might then arise in studying alkaloid-tumor cell binding, it was necessary to reduce platelet contamination to a minimum. This was done by repeated centrifugation (4–5 times) of the cells for 5 min at 300 ***g*** followed by resuspension of the cell pellet in fresh Hanks' salt solution–calf serum mixture. After the final centrifugation the cells were resuspended in Fischer's medium (Hyland, L.A., Calif., U.S.A.) containing calf serum (10%). This procedure gave a suspension of single refractile cells of which over 90% appeared to be viable on the basis of eosin exclusion; when the cell suspension was incubated with VLB at an antimitotic concentration (0.05 μg/ml) a high proportion of the cells accumulated at metaphase, indicating the presence of actively cycling cells.

Binding and release of tritiated alkaloids by platelets and tumor cells in vitro

Platelet rich plasma (*ca.* 10^9 platelets/ml), suspensions of cells isolated from rat lymphomas (*ca.* 15×10^6 cells/ml) and cultures of L5178Y mouse lymphoma cells [17] (*ca.* 5×10^6 cells/ml) were incubated at 37°C with [^{3}H]VLB and [^{3}H]VCR in stoppered siliconized glass vials rotating at 4 rev/min. At intervals throughout the incubation portions (*ca.* 100mg) of the suspensions were withdrawn into 7 cm lengths of polyethylene tubing (i.d. 0.066 in.). The tubes, sealed at one end, were centrifuged (7 min at 4300 ***g***) and the cell pellet separated from the supernatant by cutting the tubing immediately above the pellet [10, 11]. Both pellet and supernatant were radioassayed as described below. The pellet radioactivity was corrected for the small amount of supernatant radioactivity present in the pellet fraction; this correction was less than 5% of the radioactivity present in the supernatant.

When it was necessary to restore platelets to alkaloid free plasma, the suspensions were centrifuged for 20 min at 1700 ***g***, the supernatants carefully removed and the packed cells then gently resuspended in the original volume of alkaloid free, citrated plasma (pH 6.5). The radioactivity in the very small amount of supernatant retained in the pellet accounted for less than 2% of the total radioactivity in the pellet. Platelet losses during the procedure were low (less than 5%). When restoring tumor cells to alkaloid free medium, the cell suspensions were diluted with an equal volume of alkaloid free medium, centrifuged for 5 min at 300 ***g*** and the packed cells resuspended in the original volume of alkaloid free medium. Residual supernatant radioactivity accounted for less than 1% of the pellet radioactvity. Cell losses were small (less than 5%).

Radioassay methods

For the radioassay of blood, blood fractions, platelets, cells and supernatants, samples (up to 100 mg) were incubated in scintillation vials with 0.5 ml of 1N NaOH at 37°C for 24 hr and then if necessary decolorized with hydrogen peroxide [10]. Ten millilitres of PCS solubilizer (Amersham/Searle, Illinois, U.S.A.) containing 4% glacial acetic acid were then added to the vials and the radioactivity determined by liquid scintillation counting. Duplicate samples were counted to an accuracy of ±3% at the 95% confidence level and corrected for quenching.

The percentage of the radioactivity in a sample which was due to [^{3}H]VLB or [^{3}H]VCR was determined by isotopic dilution analysis as follows. A known amount of the sample was homogenized at pH 2–3 with a large known excess of purified carrier VLB or VCR. After removing small aliquots for assaying the total radioactivity in the sample, the alkaloid in the rest of the homogenate was extracted into benzene at pH 7 and then purified chromatographically using at least 3 systems. The latter

included elution from columns of alumina, cellulose phosphate or carboxymethyl cellulose using procedures already described [10. 11. 14] and also thin layer chromatography on alumina (Eastman Kodak No. 6063) run with acetone or ethylacetate/*iso*amyl alcohol (10:1) and on silica gel (Eastman Kodak No. 6060) run with acetone or ethylacetate/ethanol (1:1). In running the columns, multiple fractions were collected during the period when the carrier was leaving the column in order to verify that the radioactivity elution profile accurately matched that of the optical density [14]. The specific radioactivity of the recovered carrier alkaloid was determined from its radioactivity and u.v. absorption, and used to calculate the percentage of radioactivity in the original sample which was due to [^{3}H]VLB or [^{3}H]VCR.

RESULTS

Radioactivity in blood and blood fractions of rats given [^{3}H]*VLB and* [^{3}H]*VCR*

The levels and distribution of radioactivity in the peripheral blood of rats given single i.p. injections of [^{3}H]VLB and [^{3}H]VCR at a nonacutely-toxic dose (0.25 mg/kg) are shown in Fig. 2. The whole blood radioactivity profiles of the 2 alkaloids (Fig. 2A) have certain features in common. For example, in both cases the radioactivity appeared in the blood almost immediately after the injection, rose sharply to a peak value and then declined rapidly to much lower values. However, marked differences were also found: while the levels of radioactivity in the blood of rats given [^{3}H]VLB were initially substantially higher than those in the blood of rats given [^{3}H]VCR, in the case of VLB they fell much more extensively and after 8 hr were well below those in the blood of rats given [^{3}H]VCR.

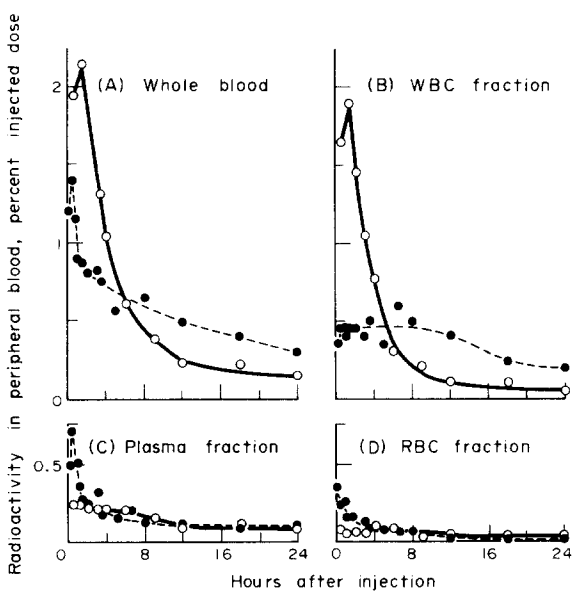

Fig. 2. Distribution of radioactivity in peripheral blood of rats given i.p. injections of [^{3}H]*VLB* (0.25 mg/kg; 3.5 × 10^8 dis/min/kg) *and* [^{3}H]*VCR* (0.25 mg/kg; 4.0 × 10^8 dis/min/kg). *Plasma, WBC and RBC fraction were obtained by centrifuging citrated tail blood samples at* 4300 *g* ("*Methods*"). *Radioactivity in whole blood* (*A*), *WBC fraction* (*B*), *plasma fraction* (*C*) *and RBC fraction* (*D*) *is expressed as percent of injected radioactivity, taking blood weight as* 7.5% *of body weight.* [^{3}H]*VLB*, ○——○, [^{3}H]*VCR*, ●--●. *Points are averages of results from at least* 3 *rats.*

A comparison of the labeling of the WBC fraction (Fig. 2B) shows that in the first few hours after the injection of [^{3}H]*VLB* this fraction was much more highly labeled than after the injection of [^{3}H]VCR. However, as time progressed this situation was reversed; in rats injected with [^{3}H]VLB the WBC radioactivity decreased sharply from its initial peak value, whereas in rats given [^{3}H]VCR the WBC radioactivity remained essentially constant for about 12 hr and thereafter declined only slowly. The labeling of the plasma fraction (Fig. 2C) was very similar for the 2 alkaloids except for a period of about 1 hr immediately after the injection. In this period the plasma radioactivity levels in the rats given [^{3}H]VCR were substantially higher than in those given [^{3}H]VLB. The levels of radioactivity in the RBC fraction (Fig. 2D) were very low except for a short period immmediately following the injection of [^{3}H]VCR; the temporary higher levels of [^{3}H]VCR radioactivity in this fraction are probably due to small amounts of entrapped plasma.

It can be seen from Fig. 2 that the WBC and plasma fractions together account for most of the radioactivity in the blood after the administration of either VLB or VCR. In the first 8–10 hr after injecting [^{3}H]VLB most of the circulating radioactivity was present in the WBC fraction. With VCR on the other hand, a substantial proportion of the circulating radioactivity was also present in the plasma, especially in the first few hours, but as time progressed most of the radioactivity was confined to the WBC fraction. It appears from the results that the differences betwen VLB and VCR in the labeling of the peripheral blood can to a major extent be accounted for by the labeling patterns of the WBC fraction.

It was shown by isotopic dilution analysis

("Methods") that, even 24 hr after the injection of [^{3}H]VCR, over 90% of the radioactivity in the WBC fraction was due to the unchanged alkaloid and furthermore using blood cell preparation methods [10], that as least 60% of the alkaloid in this fraction was associated with platelets. It appears therefore that platelets are the main cellular carriers of VCR in the peripheral blood of rats. We have already shown in a previous study [10] that in the peripheral blood, platelets are also the main cellular carriers of VLB.

Binding and release of [^{3}H]*VLB and* [^{3}H]*VCR by blood platelets* in vitro

Platelet rich plasma was incubated at 37°C with [^{3}H]VLB and [^{3}H]VCR at various concentrations. The binding of the alkaloids by platelets at 2 alkaloid concentrations is shown in Fig. 3. Very similar results were obtained at alkaloid concentrations up to 2 μg/ml. The lower concentration in Fig. 3 (0.05 μg/ml) is of the same order as that found in the plasma of rats given subacutely-toxic doses of VLB [13]. As found in a previous study [11], [^{3}H]VLB was rapidly taken up by platelets and in about 1 hr the amount of platelet bound alkaloid had reached a plateau. [^{3}H]VCR was bound by platelets at a much lower rate than VLB but eventually (after 8–10 hr) reached the same plateau as VLB at a given concentration. When the plateaus were reached, a substantial portion of the total alkaloid present had been taken up by the platelets, e.g. *ca.* 70 and 50% at alkaloid concentrations of 0.05 and 0.44 μg/ml, respectively. This demonstrates the marked ability of the platelets to concentrate the alkaloids from the plasma.

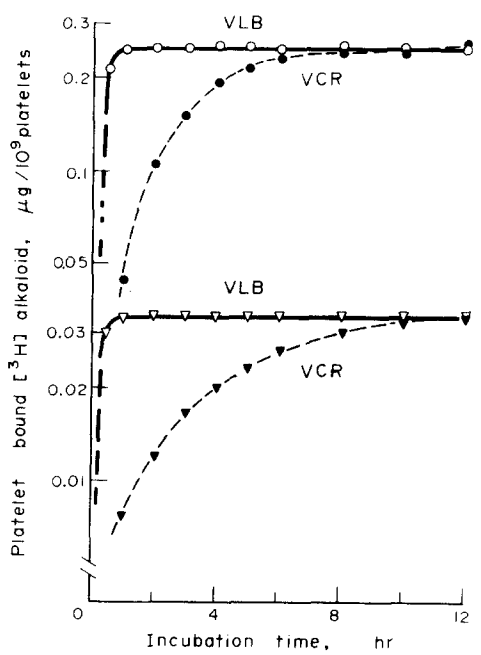

Fig. 3. Binding of [^{3}H]*VLB and* [^{3}H]*VCR by platelets* in vitro. *Aliquots of PRP containing* ca. 10^9 *platelets*/ml *were incubated at* 37°C *with* [^{3}H]*VLB* (0.44 μg/ml. ○——○; 0.05 μg/ml, ▽——▽) *and* [^{3}H]*VCR* (0.44 μg/ml. ●--●; 0.05 μg/ml, ▼--▼). *The amount of platelet bound alkaloid was determined at the times shown on centrifuged samples* ("*Methods*").

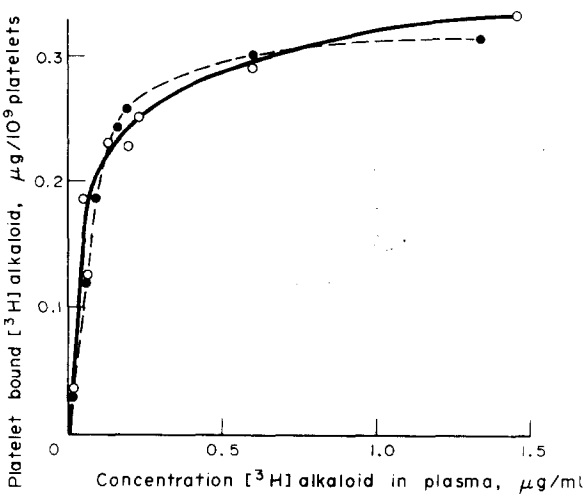

Fig. 4. Plots of amounts of [^{3}H]*VLB and* [^{3}H]*VCR bound at equilibrium by platelets vs concentrations of unbound alkaloid in the plasma. Aliquots of PRP* (*containing* 10^9 *platelets*/ml) *were incubated with the tritiated alkaloids at various concentrations. The amounts of platelet bound alkaloid and the alkaloid concentrations in the plasma were determined at intervals* ("*Methods*") *until equilibrium was established* (*usually within* 2 hr *for VLB and* 8–10 hr *for VCR; see Fig.* 3). *VLB*, ○——○; *VCR*, ●--●.

In Fig. 4 the amounts of alkaloid bound by platelets when a plateau had been reached (*ca.* 2 hr for VLB, 8–10 hr for VCR) are plotted against the concentrations of unbound alkaloid in the plasma. It can be seen that at the higher alkaloid concentrations the platelets became saturated with the alkaloids and also that over the entire concentration range the curves for VLB and VCR are essentially identical. Since the platelets bound equal amounts of VLB and VCR at saturation, it appears that the same number of binding sites are available for each alkaloid. Furthermore the close similarity in the curves indicates that the equilibrium association constants of the 2 alkaloids for the platelets are very similar.

When platelets which had taken up VLB and VCR were restored to alkaloid free plasma, there was a considerable difference in the ease with which the 2 compounds were released (Fig. 5). Platelets labeled with

[^{3}H]VLB readily released the alkaloid until equilibrium was once more established between the VLB bound by the platelets and that in the plasma (Fig. 5, upper panel). When resuspended for a second time in alkaloid free plasma, the platelets released

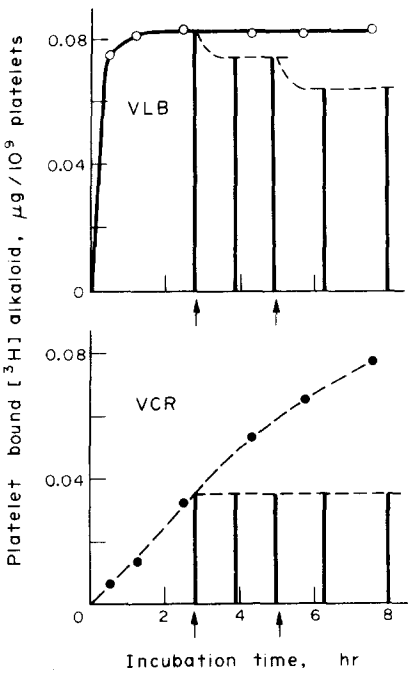

Fig. 5. Retention of [^{3}H]*VLB and* [^{3}H]*VCR by platelets on restoration to alkaloid free plasma. Aliquots of PRP were incubated at* 37°C *with* [^{3}H]*VLB* (0.11 μg/ml) *and* [^{3}H]*VCR* (0.11 μg/ml). *At intervals, samples were taken from the two suspensions for determination of the amounts of platelet associated alkaloid ("Methods"): VLB,* ○——○; *VCR,* ●--●. *After* ca. 3 hr (*1st arrow*) *platelets from a portion of each suspension were restored to alkaloid free plasma ("Methods") and the radioactivity retained by these platelets on further incubation was determined at* 2 *intervals (vertical bars,* hr 4 *and* 5). *At the time indicated by the 2nd arrow the platelets were once more resuspended in alkaloid free plasma and, after further incubation, again assayed for platelet associated alkaloid (vertical bars,* hr 6 *and* 8). *Platelet losses as a result of the manipulations were small (less than* 5%); *the results have been normalized for* 10^9 *platelets.*

a further amount of VLB, again establishing an equilibrium between bound and free alkaloid. The results indicate that the binding of [^{3}H]VLB by platelets is readily reversible, confirming conclusions reached in a previous study [11]. In contrast, platelets which had been labeled with [^{3}H]VCR released very little of the bound alkaloid when restored to alkaloid free plasma; even after a second resuspension in alkaloid free plasma and prolonged incubation the platelets retained essentially all the VCR initially bound (Fig. 5, lower panel).

Labeling of the WBC fraction in rats given i.v. injections of platelets prelabeled with [^{3}H]*VLB and* [^{3}H]*VCR*

The finding that platelets are the main cellular carriers of VLB and VCR in the peripheral blood of rats suggested that the differences in the labeling patterns of the WBC fractions in rats given the tritiated alkaloids (Fig. 2B) might be related to the differences in the alkaloid binding properties of platelets as found in the *in vitro* studies (Figs. 3 and 5). One aspect of this suggestion was investigated by giving rats intravenous injections of platelets which had been prelabeled *in vitro* with [^{3}H]VLB or [^{3}H]VCR and then determining the levels of radioactivity in the blood and its distribution among the blood fractions for 1–2 days. During this period essentially all the blood radioactivity was found to be concentrated in the platelet containing WBC fraction; the amounts of radioactivity in the plasma and RBC fraction were negligible. Figure 6 shows the percentage of injected radioactivity present in the WBC fraction of the rats, calculated on the basis that blood volume is 7.5% of body weight. In the first few min after the injection of platelets labeled with [^{3}H]VLB or [^{3}H]VCR, most of the injected radioactivity was in each case found to be present in the WBC fraction. Since the platelets were injected into the jugular vein and the blood was

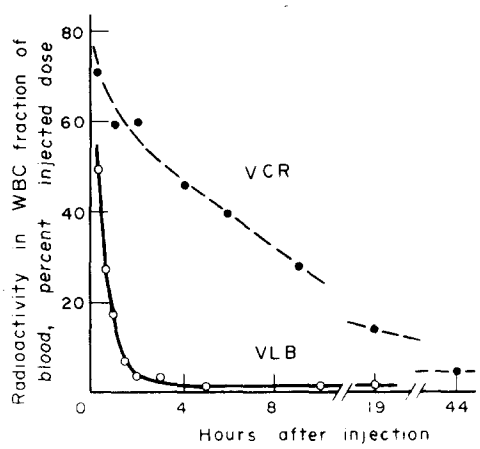

Fig. 6. Radioactivity of WBC fraction of peripheral blood of rats given i.v. injections of platelets prelabeled with [^{3}H]*VLB and* [^{3}H]*VCR. Platelets were prelabeled by incubating PRP at* 37°C *for* 3 hr *with* [^{3}H]*VLB* (0.3 μg/ml) *and* [^{3}H]*VCR* (0.9 μg/ml), *collected by centrifugation and resuspended in small volumes of alkaloid free plasma. Platelets* (ca. 3×10^9) *labeled with* [^{3}H]*VLB* (0.47 μg) *and* [^{3}H]*VCR* (0.48 μg) *were injected into the jugular vein of rats. The radioactivity in the WBC fraction of the total blood of the rats (calculated on the basis that blood weight is* 7.5% *of the body weight) is given as a percentage of injected radioactivity (means of* 2 *determinations). VLB,* ○——○; *VCR,* ●--●.

sampled from the tail, it appears that the platelets had been rapidly dispersed in the circulation. The rates at which the levels of WBC radioactivity (and hence also the levels of radioactivity in the blood) declined from their initial high values were markedly different for the 2 alkaloids. Thus after the injection of [^{3}H]VLB labeled platelets the WBC radioactivity declined very rapidly and within 2 hr only a few per cent of the injected radioactivity remained in the blood; in contrast, the levels of radioactivity in the WBC fraction after the injection of [^{3}H]VCR labeled platelets declined much more slowly and even after 10 hr over 25% of the injected radioactivity was still present in the circulation. It may be noted that the decline in the radioactivity of the WBC fractions will reflect not only the rates at which the [^{3}H]alkaloids are released from the platelets but also the loss of alkaloid labeled platelets from the circulation, e.g. as a result both of normal platelet turnover and the exchange of platelets in the circulation with those elsewhere in the body. On the assumption that the rate of platelet loss from the peripheral blood is the same for platelets labeled with [^{3}H]VLB as for platelets labeled with [^{3}H]VCR, it seems very probable that the difference between the 2 alkaloids in the decline of the WBC radioactivity (Fig. 6) is due largely to a ready release of VLB and a contrasting tenacious retention of VCR by platelets *in vivo*.

Binding and release of [^{3}H]*VLB and* [^{3}H]*VCR by tumor cells* in vitro

The binding and release of [^{3}H]VLB and [^{3}H]VCR by tumor cells *in vitro* was studied using suspensions of cells isolated from rat Node lymphomas and suspensions of cultured L5178Y mouse lymphoma cells. Figure 7 shows the labeling of the cells isolated from rat lymphomas when incubated with [^{3}H]VLB and [^{3}H]VCR at two concentrations, the lower of which (0.05 μg/ml) is in the range of that found in the plasma of rats given a therapeutically effective dose of VLB [13]. Both alkaloids were taken up by the cells from the medium at rates which for each alkaloid were proportional to its concentration. At a given alkaloid concentration, VLB was bound by the cells much more quickly than VCR. Thus, whereas the amount of [^{3}H]VLB bound by the cells reached a plateau in *ca.* 50 min, at least 5 hr elapsed before the amount of VCR bound approached a plateau. The

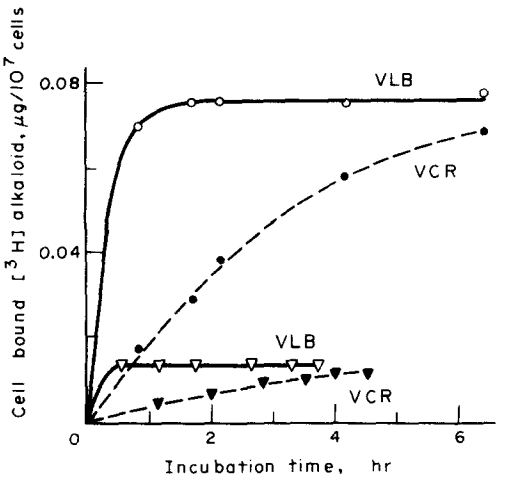

Fig. 7. Binding in vitro *of* [^{3}H]*VLB and* [^{3}H]*VCR by cells isolated from rat lymphomas. Aliquots of cell suspensions prepared from rat lymphomas* (ca. 15×10^6 *cells*/ml: "*Methods*") *were incubated at* 37°C *with* [^{3}H]*VLB* (0.46 μg/ml, ○——○; 0.05 μg/ml, ▽——▽ *and* [^{3}H]*VCR* (0.46 μg/ml, ●--●; 0.05 μg/ml, ▼--▼). *The amount of cell bound alkaloid was determined at the times shown on centrifugated samples* ("*Methods*").

amount of VCR eventually bound, however, quite closely approximated the amount of VLB bound at the same concentration. Isotopic dilution analysis showed that the cell associated radioactivity was due almost entirely to the unchanged alkaloids, e.g. after 5 hr incubation with the alkaloids, 85 and 95% of the cell bound radioactivity was due to [^{3}H]VLB and [^{3}H]VCR, respectively.

When isolated lymphoma cells, which had been prelabeled by incubation with [^{3}H]VLB and [^{3}H]VCR, were restored to alkaloid free medium and further incubated, they released the two alkaloids at markedly different rates. The results of a typical experiment are shown in Fig. 8. The cells which had been prelabeled with [^{3}H]VLB released a substantial amount of the cell bound alkaloid each time they were reincubated in fresh alkaloid free medium and after 6 such treatments the cells retained only 5% of the alkaloid initially taken up. In contrast, the cells prelabeled with [^{3}H]VCR released very little radioactivity after each reincubation in alkaloid free medium and even after 6 medium changes the cells retained over 75% of the [^{3}H]VCR originally bound. Figure 8 also shows that even if cells have initially taken up much more VLB than VCR, on lowering the extracellular alkaloid concentration VLB is released so readily that eventually the amount of VCR retained by the cells can greatly

exceed that of VLB. For example, although on incubation for 100 min with the [^{3}H]alkaloids, the tumor cells took up only one-third as much VCR as VLB, after 6 reincubations in alkaloid free medium they retained 6 times as much VCR as VLB.

Cultured L5178Y mouse lymphoma cells behaved very similarly to the cells isolated from the rat lymphomas with respect to the uptake and release of the tritiated alkaloids, i.e. [^{3}H]VLB was taken up quickly and readily released; [^{3}H]VCR was taken up slowly and released very slowly (data not included).

DISCUSSION

The present study has shown that VLB and VCR differ markedly both in the pharmacokinetics of their distribution in the peripheral blood of rats and in their binding by cells. The differences between the alkaloids in their uptake and release by cells seem to be particularly important since they appear to provide explanations not only of the differences in the pharmacokinetic distribution of the alkaloids, but also of some of the differences in the toxic and oncolytic properties of the alkaloids.

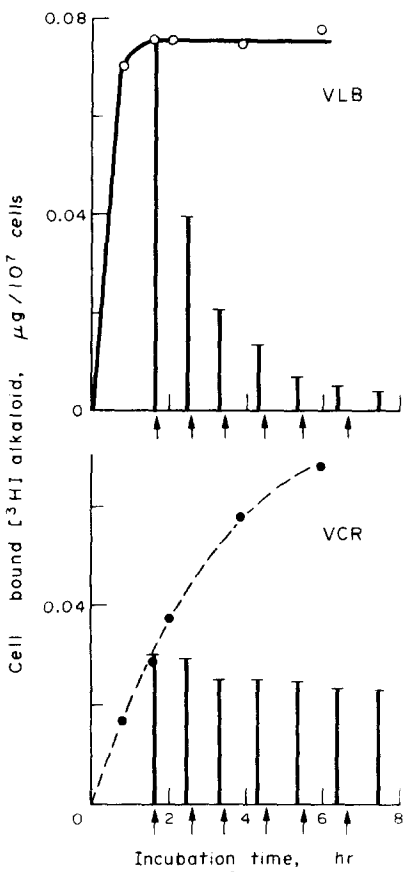

Fig. 8. Retention of [^{3}H]VLB and [^{3}H]VCR by isolated rat lymphoma cells on restoration to alkaloid free medium. Aliquots of cell suspensions prepared from rat lymphomas ("Methods") were incubated at 37°C with [^{3}H]VLB (0.46 μg/ml) and [^{3}H]VCR (0.46 μg/ml). The amounts of cell bound alkaloid were determined at intervals up to 6 hr (VLB, O——O; VCR, ●--●). After labeling the cells for 100 min a portion of each suspension was taken for determining the amounts of alkaloid retained by the cells when restored to alkaloid free medium ("Methods"). At each of the times indicated by the arrows the cells were resuspended in alkaloid free medium and incubation continued; the vertical bars indicate the amounts of alkaloid retained by the cells 50 min after each resuspension. The total loss in cell numbers resulting from the multiple resuspensions was less than 10%; the results have been normalized for 10^7 cells.

The marked differences observed in the pharmacokinetic distribution of VLB and VCR in the peripheral blood of rats (Fig. 2) can be correlated with differences in the alkaloid binding properties of blood platelets which, as shown in this and a previous study [10], are the main cellular carriers of VLB and VCR in the peripheral blood. It seems very likely, for example, that the high concentrations of VLB which are found almost immediately in the WBC fraction (Fig. 2B) and the comparatively low labeling of the plasma by [^{3}H]VLB (Fig. 2C) reflect the high rate at which platelets are able to concentrate VLB from the plasma, as found *in vitro* (Fig. 3). Furthermore, the higher levels of VCR initially found in the plasma and the relatively lower amounts of VCR in the WBC fraction are consistent with the lower rate at which platelets take up VCR as compared to VLB (Fig. 3). The rapid decline in the levels of VLB in the WBC fraction and the contrasting sustained levels of VCR in this fraction (Fig. 2B) can likewise be explained by the ease with which platelets release VLB and the tenacity with which they retain VCR, as found on restoration of labeled platelets to alkaloid free plasma *in vitro* (Fig. 5) and also *in vivo*, when platelets labeled with VLB and VCR were injected intravenously into rats (Fig. 6).

The marked differences between VLB and VCR in their rates of uptake and release by cells were found not only with blood platelets but also with cells isolated from rat lymphomas and cultured L5178Y mouse lymphoma cells. Thus all the cells took up VLB much more quickly than VCR (Figs. 3 and 7) and on restoration to alkaloid free medium readily released VLB but tenaciously retained VCR (Figs. 5 and 8). In view of the similarity in the alkaloid binding properties of cells of such diverse types as platelets (with no nucleus and comparatively simple metabolism) and replicating tumor cells, it seems likely that the mechanisms involved in the binding of the

alkaloids by cells—including their ability to differentiate between the 2 alkaloids—are common to a variety of cell types.

The use of platelets as comparatively simple models for studying the interaction of Vinca alkaloids with their cellular receptors has been indicated by a previous study [11] in which it was shown that in binding [^{3}H]VLB, platelets behave as if they have saturable receptors with a high affinity for the alkaloid; the displacement of platelet bound [^{3}H]VLB by non-radioactive VCR could tentatively be explained by a kinetic model in which VCR competed for the same binding sites as VLB but at a much lower rate. The present study, in which [^{3}H]VCR as well as [^{3}H]VLB has been used, provides strong support for this model. Thus it shows that even though, at a given alkaloid concentration, platelets may eventually take up as much VCR as VLB, they bind VCR at a much lower rate than VLB (Fig. 3). The finding that at saturation platelets bind equal amounts of each alkaloid, together with our previous observation that [^{3}H]VLB is displaced by VCR [11], strongly suggests that in platelets the 2 alkaloids do in fact have the same binding sites. In this connection it may be noted that Bleyer *et al.* [18] have concluded on the basis of competition studies that VLB and VCR have common binding sites in L1210 leukemia cells. It also appears that while VLB and VCR differ markedly in the rates at which they are taken up by platelets, they nevertheless have similar equilibrium association constants for platelets as indicated by the results in Figs. 3 and 4. VLB and VCR appear also to have very similar equilibrium association constants for cells isolated from rat lymphomas and for L5178Y cells, since, at a given alkaloid concentration, the cells eventually take up about the same amounts of each alkaloid (Fig. 7). It is of considerable interest that VLB and VCR should differ so markedly in their rates of uptake and release by platelets and tumor cells even though they have similar equilibrium association constants for the cells and apparently common receptors.

There is substantial evidence from other work that a major receptor for the biologically active Vinca alkaloids, including VLB and VCR, is "tubulin"—the protein subunits of cellular microtubules [5–7]. Although in the present study the specific nature of the cellular alkaloid receptors has not been investigated, it may be noted that exposure of the cells to the alkaloids resulted in pronounced disturbances in microtubular structures—indicative of tubulin–alkaloid interaction—i.e. disruption of the mitotic spindle in the tumor cells and of the circumferential bundle of microtubules in the platelets [10, 19]. The binding of VLB and VCR to isolated tubulin in certain respects resembles their binding to intact cells, e.g. both alkaloids have a high affinity for tubulin with association constants that are of the same order [20, 21] and they also appear to compete for the same binding sites on the tubulin [22, 23]. In spite of these similarities, however, the intact cells and tubulin differ markedly in the rates at which they bind VLB and VCR. Thus whereas VCR is bound much more slowly than VLB by platelets and tumor cells (Figs. 3 and 7), both alkaloids have been reported to bind rapidly to isolated tubulin, reaching equilibrium after only a few minutes incubation, at which point equal amounts of the compounds were bound [20]. This suggests that if the alkaloid binding properties of tubulin in intact cells are the same as *in vitro*, then the different rates at which VLB and VCR are taken up by cells are not determined primarily by differences in their binding to tubulin but are due instead to a rate controlling process which differentiates between the 2 alkaloids and which is not directly related to the interaction of the alkaloids with tubulin. It is tempting to speculate that the difference between VLB and VCR in their uptake (and possibly their release) by cells may stem from differences in the rates at which they are transported across the plasma membrane.

The pharmacokinetic distribution of VLB and VCR in the blood and the binding of the alkaloids by cells are, almost certainly, important variables mediating the toxic and oncolytic effects of these compounds. This raises the question of the extent to which *differences* in the latter are related to differences in the levels and distribution of VLB and VCR in the peripheral blood (Fig. 2). Although the levels of VCR in the plasma were for a short period substantially higher than those of VLB (Fig. 2C), it appears unlikely that during this period cells will accumulate even as much VCR as VLB in view of the lower rate at which they take up VCR. It is doubtful therefore if the transient higher levels of

VCR in the plasma account to a major extent for the greater toxicity and oncolytic potency of VCR. Regarding the transport of the alkaloids in the WBC fraction, it is possible that in the case of VLB the platelets may, as suggested elsewhere [11], play a role in the delivery of this alkaloid to tissues, in view of the readiness with which they take up and release VLB. On the other hand it is rather doubtful if platelets play a significant role in the delivery of VCR, in view of the tenacity with which they retain VCR. While the overall effect of the differences in the transport of VLB and VCR in the blood is not entirely clear, they do not appear to provide an adequate explanation of the differences in the toxic and oncolytic properties of the 2 alkaloids.

While, as indicated above, there is substantial evidence that the biological effects of VLB and VCR stem primarily from their binding to tubulin [5–9], the relatively minor differences in the interaction of the alkaloids with tubulin do not appear to account for the marked differences in the toxic and oncolytic properties of the compounds. In fact, Himes *et al.* [24] have suggested that the latter are likely to be determined by processes other than the interaction of VLB and VCR with tubulin *per se*, since the alkaloids not only have very similar tubulin binding properties but are also equally effective in inhibiting the polymerization of tubulin *in vitro*. From results in the present study it seems likely that at least a partial explanation of the greater toxicity and oncolytic potency of VCR may lie in the markedly greater tenacity with which cells retain VCR. Thus even though cells, on exposure to the alkaloids, may accumulate less VCR than VLB (e.g. as a result of the lower rate at which VCR is taken up), they will, in the face of falling extracellular alkaloid levels, retain VCR much longer and eventually in greater amounts than VLB (Fig. 8). It may be anticipated that when sensitive cells are exposed to the alkaloids for short periods, as may occur *in vivo*, VCR, as a result of its retention by cells, will continue to exert its toxic effects long after its extracellular levels have fallen to insignificant values; the effects of VLB on the other hand are likely to be less persistent in view of the readiness with which this alkaloid is released by cells when the extracellular alkaloid levels decline (Fig. 8). The differential in the retention of VLB and VCR by cells may assume special significance when asynchronously replicating cells are exposed to the alkaloids. Replicating cells are particularly sensitive to the alkaloids at mitosis and if briefly exposed to the compounds at less sensitive phases of the cell cycle, may retain much more VCR than VLB by the time they reach mitosis. VCR may also, in view of its more sustained retention by cells, have a more adverse effect than VLB on the recovery of cells from microtubular damage, perhaps accounting for its greater neurotoxicity.

In view of the readiness with which VLB is released by cells it may be expected that its toxic effects will be more dependent than those of VCR on the maintenance of elevated alkaloid levels in the immediate environment of cells. In this connection it is of interest that whereas, *in vitro*, cells isolated from rat lymphomas readily release VLB (Fig. 8), nevertheless in the animal the lymphoma has been found to retain the alkaloid long after the levels of VLB in the circulation had fallen to very low levels; it was suggested that the continuing presence of VLB in the tumor might be an important factor underlying the high sensitivity of the lymphoma to treatment with the alkaloid [13]. The mechanism of retention of VLB by the lymphoma *in vivo* is not yet known but may be related to the lymphoid character of the tumor tissue [13]. The difference in the retention of VLB *in vitro* by isolated cells and *in vivo* by the lymphoma raises the possibility that there may be more than one mechanism for maintaining elevated alkaloid levels in "target" tissues, e.g. retention by cells *per se* as found for VCR, and retention by virtue of some special property of the tissue as suggested for VLB in the lymphoma. The existence of alternative mechanisms could help to explain why VLB has a more limited spectrum of oncolytic activity than VCR, and why, for example, it is useful in the treatment of certain lymphomas (e.g. Hodgkin's disease, Nb strain rat lymphomas) but is much less effective than VCR in treating leukemias. Further studies on the mechanism of action of the Vinca alkaloids both at the cellular level and *in vivo* are clearly required.

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