Paramitotic Effects of Colchicine: Further Studies of the Mast-Cell Assay (32654)

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Morphological alterations in nonmitosing cells are induced by colchicine administration (1–4). These alterations are most readily detected in the tissue fluids in which the normally spherical free-floating cells assume a nonspherical shape after colchicine administration. Peritoneal fluid mast cells are most conspicuously affected. In young adult rats these are spherical and their nucleus is located at the center of the cytoplasm, with the numerous specific granules evenly distributed (4). The colchicine effect first involves displacement of the nucleus to the periphery, with concomitant displacement of the granules in that area; part of the nucleus appears devoid of cytoplasm by brightfield microscopy. However, with differential interference contrast optics, this aspect of the nucleus was seen to be the site of extensive hyaloplasmic activity (8), a fact supported by electron-microscopic observations (unpublished personal observations). Later stages of the response, especially at higher doses, involve elongation of the cytoplasm with local constrictions leading to a beaded appearance.

The response is so reproducibly elicited, and peritoneal fluid is so readily handled, that peritoneal mast cells of the rat have been utilized to assess activities of some colchicine derivatives (3,4). Podophyllotoxin (and its derivatives) (5) and vinblastin (3, 6) both of which elicit a colchicine-like blockage of mitosis by interfering with the achromatic apparatus, evoke paramitotic effects indistinguishable from those of colchicine. The solubilities of these drugs differ significantly and thus required the use of a number of different solvents. Because the solvent may alter the rate of absorption of the substance tested, it is important to determine how these solvents affect the response.

In this study, three factors were examined: (1) the time course of the response as a function of dosage, (2) the effect of altering the solvent, and (3) a comparison of subcutaneous versus intraperitoneal routes of administration.

Materials and Methods. Male rats of the Holtzman Sprague-Dawley strain, 39–44 days old and weighing 90–100 gm were used and were given food and water *ad libitum* throughout the experiments. Colchicine was obtained from a commercial source (S.B. Penick & Co., N.Y., USP grade). It was used as is, except for the experiments concerned with intraperitoneal administration. For these, the drug was chromatographed and recrystallized twice from ethyl acetate by the method of Ashley and Harris (7). Prior to intraperitoneal injection, the animal was anesthetized lightly with ether, a small skin incision was made along the midventral line, and the abdominal musculature was exposed. The muscles were raised with forceps and the needle (26G, ¼ inch) was inserted carefully in order to insure that the drug was delivered into the cavity. The abdomen was then massaged immediately for about 5 sec and the skin incision was closed with a Michel clip.

At predetermined intervals, rats were anesthetized with ether and killed by decapitation. The body cavity was exposed and a sample of peritoneal fluid was aspirated into a white blood cell pipette. This sample was diluted either with Randolph's fluid (8) or with a modification thereof consisting of: methylene blue chloride, 10 mg; propylene glycol, 10 ml; distilled water, 10 ml; acetic acid, glacial, 0.1 ml; and formaldehyde, 37%, 0.2 ml.

The percentages of affected cells were then determined under microscopic examination (hemacytometer; 300×).

Three sets of experiments were performed. First, the time course of the response was established for a single subcutaneous injection of colchicine. Two higher doses were then tested and samples obtained 24 hours after

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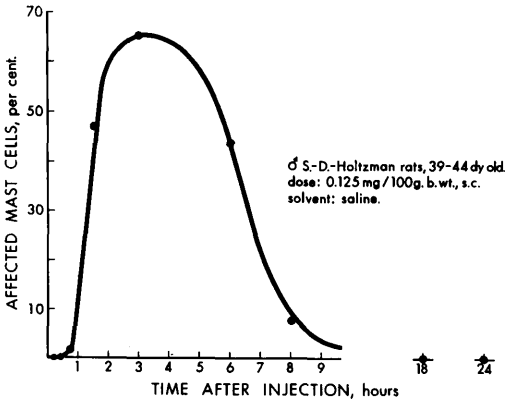

FIG. 1. Response of peritoneal fluid mast cells as a function of time following a single subcutaneous injection of colchicine. Each point represents the average for eight animals.

injection, a time interval when the mast cells had returned to normal in the animals given the lowest dose.

Second, a single dosage of 0.125 mg of colchicine/100 gm body weight was injected subcutaneously and the response was assessed after 3 hours. The drug was dissolved in one of four solvents: (1) 0.9% sodium chloride aqueous, (2) distilled water, (3) ethanol, 50% aqueous, and (4) propylene glycol, 50% aqueous.

Third, rats were injected intraperitoneally with graded dosages of colchicine ranging from 0.00001 mg to 0.2 mg/100 gm body weight and the response was measured 4 hour later. Concomitantly, for comparison purposes, another group of animals was injected subcutaneously with 0.1 mg colchicine/100 gm body weight. Finally, a uniform dose of 0.2 mg colchicine per rat (equivalent to from 0.21 to 0.23 mg/100 gm body weight) in 0.01 ml saline was injected intraperitoneally with a microsyringe and the response was assessed 4–6 min after injection. The volume injected was small in order to avoid excessive dilution of the peritoneal fluid.

Results. The effects of a single subcutaneous injection of 0.125 mg colchicine in saline/100 gm body weight are depicted in Fig. 1. No affected cells were recognized at either 11 or 22 min after injection, and only about 2% were seen at 45 min. At 1 hour almost 50% of the cells were affected and a peak of some 65% of morphologically altered cells occurred at 3 hours. Subsequently, the relative numbers of altered cells declined steadily and by 18 hours the mast cell population appeared entirely normal. In general, the degree to which the cells were affected paralleled the percentages involved. When the larger doses of colchicine were given, however, some affected cells were still encountered 24 hours later: at a dose of 0.175 mg colchicine/100 gm body weight, one animal out of 10 sampled had 0.5% affected mast cells and at a dose of 0.2 mg colchicine/100 gm body weight all of 10 rats sampled still had affected mast cells, whose numbers averaged 0.91%.

Table I summarizes the effects of various solvents when a standard dosage of colchicine was injected subcutaneously. Statistical analysis revealed no significant differences between the groups tested ($p > .05$). Furthermore, under microscopic examination, the mast cells appeared equally altered in all groups. In contrast to these findings, gross examination of the injection sites after 3 hours revealed differences in the local tissue reaction. Whereas saline or distilled water did not elicit any noticeable changes, ethanol and propylene glycol evoked a local inflammatory response. With propylene glycol, a mild inflammatory effect with slight hemorrhage was seen. Ethanol evoked the most severe reaction which included appreciable hemorrhage and some caseous material surrounded by a gelatinous and edematous matrix.

Affected mast cells were detected soon after intraperitoneal injection of colchicine. In a group of seven rats sampled 4–6 min after injection, an average of 36.0% of the mast cells were already affected although the mor-

TABLE I. Effects of Solvent on Standard Subcutaneous Dose of Colchicine at 3 Hours Postinjection.

Solvent	No. of animals in group	% Affected mast cells[a]
Saline	16	60.7 ± 4.0
Distilled water	16	60.1 ± 3.2
Propylene glycol, 50% aqueous	18	54.2 ± 4.4
Ethanol, 50% aqueous	16	46.2 ± 5.9

[a] Mean ± SEM.

TABLE II. Per cent Affected Mast Cells 4 Hours after a single Injection of Colchicine.

Dose, mg/100 gm body weight	Route	% Affected mast cells	Average[a]
0.1	s.c.	1.8, 6.9, 1.3, 0, 61.6	14.3 ± 11.9
0.2	i.p.	81.2, 82.1, 86.0	83.1
0.1	i.p.	88.3, 80.4, 78.4, 97.7, 83.3	85.6 ± 3.4
0.05	i.p.	81.8, 91.0, 50.0	74.3
0.025	i.p.	78.0, 58.9, 90.1	75.7
0.0125	i.p.	82.3, 78.5, 72.2	77.7
0.00625	i.p.	80.8, 91.8, 77.9	83.5
0.00313	i.p.	84.5, 92.8, 83.1	86.8
0.0016	i.p.	79.6, 67.0, 76.1, 71.8, 70.7, 80.4	74.3 ± 2.2
0.0008	i.p.	66.8, 48.9, 64.7, 57.9, 72.6, 79.9	65.1 ± 4.4
0.0004	i.p.	4.0, 8.0, 11.8, 0.7, 11.8, 31.6	11.3 ± 4.4
0.0002	i.p.	0, 0.2	0.1
0.0001	i.p.	0, 0, 0	0
0.00001	i.p.	0.3, 0, 0.2	0.2

[a] Standard error of the mean is indicated where appropriate.

phological changes were minimal. Four hours after peritoneal injection the degree to which the mast cells were altered was fully comparable to that seen after subcutaneous administration of the drug. The peritoneal route, however, was far more efficacious than the subcutaneous one: at a dose of 0.1 mg of colchicine/100 gm body weight, only 14.3% of the mast cells were affected after subcutaneous injection as compared with 85.6% following intraperitoneal injection. Individual values obtained 4 hours after intraperitoneal injection are listed in Table II. Note that only one determination exceeds 93%. Data for the dosage range 0.0001–0.0125 mg colchicine/100 gm body weight are presented in graphical form on log-probability paper (Fig. 2).

Discussion. The paramitotic, cytomorphological changes elicited by colchicine are transient. It is probable that they are reversible. It has been shown by Allen (10) that colchicine actually inhibits inception of mitosis in mast cells, and thus it appears unlikely that a new population arises by cell division. Furthermore, mast-cell numbers are not significantly different 24 hours after injection of the drug than they are in intact rats. Since differentiation of fully granulated mast cells *de novo* after eradication from the peritoneal cavity of the rat requires some 45 days (11), it is also unlikely that a new population could arise from undifferentiated precursors within 18–24 hours after colchicine injection. It might be argued that mast cells in the surrounding connective tissues of the peritoneal cavity are immune to the drug and that these are shed into the peritoneal fluid and replace the colchicine-altered cells. This, too, is unlikely because mesenteric mast cells are similarly affected by the drug (1, 12). Lastly, experiments in progress indicate that colchicine-altered mast cells can revert to their normal appearance *in vitro*.

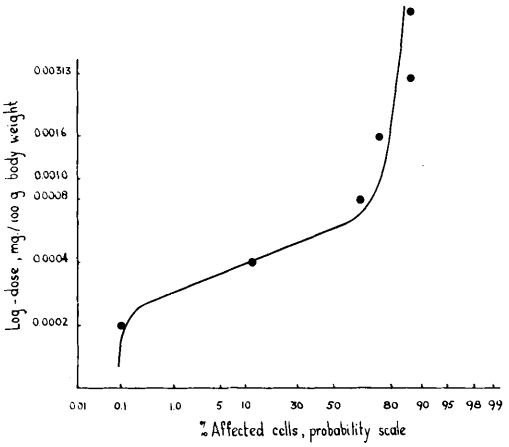

FIG. 2. Log-dose-probability plot for intraperitoneal colchicine injections. This curve should be compared to a similar curve for subcutaneous administration of colchicine already published elsewhere (3). For the concentration range suitable for assay purposes, the slope of both curves is identical.

A number of factors could affect the time required to elicit the effect on mast cells following colchicine administration. These include (1) the site of injection, (2) the rate of absorption from the injection site, (3) the time required for interaction between the drug and secondary factors, and (4) the time required by the cell to undergo morphologic changes once it has received the final common stimulus. The present experiments indicate that the lag time was shortened to as little as 4–6 min for a definite effect when the drug was instilled *in situ* as compared to 45 min for a minimal effect after subcutaneous injection. This suggests a possible direct effect of colchicine on the cells. With a variety of solvents which lead to variable degrees of local reaction, comparable mast-cell responses were nevertheless obtained and thus the solvent is not critical. The fact that the maximum response after intraperitoneal injection was generally under 90% even for the high dose of 0.2 mg/100 gm body weight, whereas the maximum obtained after subcutaneous injection was usually over 97% (3), suggests that endogenous substances not normally present in peritoneal fluid may be required. This problem is currently under investigation.

Finally, even when all conditions for evoking mast-cell changes are fulfilled, a significant time is required by the cell to alter itself morphologically. Time-lapse observations (8) have indicated that this is a progressive and continuous process in which cytoplasmic streaming plays a major role and in which all parts of the cell are involved simultaneously. The short-term experiments indicate that about 4–6 min is required for the nucleus to become relocated from a central to a peripheral position, i.e., for the cell to assume the earliest operationally detectable criterion that it has become "affected."

From the assay standpoint, the shape of the curve in Fig. 1 indicates that the 3-hour interval between injection and sampling is satisfactory since the response reaches a maximum at about that time. Since similar values are obtained from about 2½–4½ hours after injection, it may be advisable to sample at a somewhat later time, perhaps at 3½ or 4 hours postinjection, to avoid a point so close to the shoulder of the response curve, even though any error would be relatively small at this point of the curve.

It is interesting that solvent effects should be so slight even in the face of obvious differences in local tissue damage at the injection site. The local irritation may have developed relatively late in the reaction, perhaps too late to affect substantially the early circulating titers of the drug. In view of the known rapid responsivity of the subcutaneous vascular bed, this does not seem too likely, although it remains a distinct possibility. Rather, it seems reasonable to assume that the obvious differences in local tissue damage at the injection site should be reflected in different absorption rates. If this were indeed the case, it would suggest that absorption rates may not be of primary concern and, furthermore, that the effect is not direct, or that it requires some co-factor(s) which may impose rate-limiting steps in the reaction.

The peritoneal route of drug administration is suitable for assay purposes. The slope of the log-dose response curve obtained at 4 hours (Fig. 2) is, in fact, identical to that obtained for the assay performed with subcutaneous injections (3). In the lower part of the range suitable for assay purposes, a 10% response was obtained with a dose of 0.075 mg colchicine/100 gm body weight injected subcutaneously (3). A quantitatively similar response was obtained with only 0.004 mg/100 gm body weight of the drug administered intraperitoneally. This represents a twentyfold increase in sensitivity for the assay performed with intraperitoneal injections. Both routes are likely to prove useful for assay purposes since the fragility of mast cells will, no doubt, preclude the use of many solvents that could be used with impunity for subcutaneous injection. By either route, the mast cells are sensitive detectors of the drug. Colchicine rapidly dilutes to the total body water *in vivo* and, because it is not readily metabolized, its concentration in the body fluids remains relatively constant for several hours (13, 14). On this basis, first approximations of drug activity indicate colchicine to be effective at a body concentration of approximately 2×10^{-8} M for subcutaneous injections. This level of activity with respect to the paramitotic effects of the drug is in the range, or

somewhat less, than the concentration required to block mitosis in tissue culture and well below that needed for some other cellular effects unrelated to mitosis (15). It was observed incidentally to the present studies that the other cell types of rat peritoneal fluid often show evidence of the colchicine effect at even lower doses of the drug. Other cell types are not readily amenable to quantitative studies, however.

The lack of solvent effect may suggest that absorption rate is not limiting. Absorption may play some role, however, since a much shorter time lag is present with intraperitoneal injection. This indicates that the cell can detect the stimulus, and can respond to it very quickly. It does not rule out the possibility that this lag is also dependent on a rapid release or transfer of some necessary cofactor substance into the extracellular peritoneal-fluid environment. The discrepancy in lag times between the two routes of administration also may be compatible with the idea that the response depends on the presence of some cofactor(s) to mediate the colchicine effect. Further studies to explain the differences in lag time are obviously indicated.

A second unexplained finding is that more cells can be affected by high doses given subcutaneously than can be obtained when the drug is given intraperitoneally. This may be an artifact because anucleate fragments are not easily identified as such in the hemacytometer. Some such fragments were definitely present after intraperitoneal administration, but not after subcutaneous injections. On the other hand, it, again, may be referable to some accessory factor(s) as hypothesized above, although a mechanism for such a postulated effect does not readily come to mind. Still another alternative explanation which remains to be explored may be that the time course of the response is speeded up in the intraperitoneal assay, i.e., that the mast-cell population was already recovering at 4 hours, the time when it was sampled. If true, this would suggest that a factor (either intra or extracellular) necessary for the response may in time become exhausted. It is notable that the slope of the assay curve is the same not only for subcutaneous versus intraperitoneal administration of the drug but also for two families of chemically very different drugs, namely podophyllotoxin and vinblastin. If a systemic factor does in fact exist, it might explain these presently mysterious findings. However, the evidence offered by Midgley *et al.* (16), showing that some of the colchicine effects are referable to the cellular rather than systemic levels, will have to be reconciled with the cofactor hypothesis. A priori, there is no reason to think that these two concepts are mutually exclusive.

The extracellular cofactor hypothesis is further supported by experiments in progress which indicate that direct *in vitro* addition of colchicine to peritoneal fluid only sporadically results in the characteristic alteration of mast cell morphology.

Both *in vivo* and *in vitro* approaches are presently pursued in order to elucidate the mechanisms involved.

If demonstrable, this cofactor hypothesis may illuminate several as yet unexplained observations including: (1) the delayed death that occurs even when 1000 times the lethal dose of colchicine is administered (17); (2) the decreasing sensitivity to colchicine with aging (18); (3) the protective effect of reduced body temperature on colchicine poisoning (17); (4) the baffling similarity of effects shared by colchicine, podophyllotoxin, and vinblastin; (5) the mechanisms by which dividing cells escape the mitotic block induced by colchicine; (6) the parallelism between the nonmitotic and the mitostatic effects of colchicine (19); and (7) the mechanism of action of colchicine in gouty arthritis. It also offers a hope that an antidote for colchicine poisoning may yet be discovered.

Conclusions. The paramitotic cytomorphological effects of colchicine are transient and probably reversible. For assay purposes, the drug can be administered either subcutaneously or intraperitoneally. The latter route increases the sensitivity of the assay by about 20 times. A single sampling at 3–4½ hours is justifiable and, within reason, the choice of solvent for the drug appears of secondary importance—at least for subcutaneous injection.

It is postulated that colchicine does not act alone at the cellular level, but that it must act in concert with some extracellular (perhaps systemic) factor or factors. Some im-

plications of this hypothesis are mentioned.

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Effect of WY-5256 on Renal Hemodynamics* (32655)

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WY-5256 (4-amino-7-(2-methoxyethylamino)-*N*-(2-methoxyethyl)-2-phenyl-6-pteridine carboxamide) is a new orally active pteridine diuretic reported to have approximately the same activity and potency as hydrochlorothiazide in saline loaded rats and dogs (1).

Inasmuch as other pteridine agents appear to affect renal hemodynamics (2), the purpose of the present study was to determine the effect of this agent on renal hemodynamics and to determine if the increase in sodium excretion produced by WY-5256 could be related to such changes. Changes in renal hemodynamics were determined with an electromagnetic flow meter in conjuction with *p*-aminohippurate (PAH) and inulin clearances.

Methods. The studies were carried out on dog kidneys *in situ*. Mongrel dogs weighing 15–29 kg were anesthetized with sodium pentobarbital, 30 mg/kg. A tracheal cannula was inserted to insure free passage of air. The kidney to be utilized was exposed by a flank incision and the renal artery was cleared of the surrounding tissue.

A flow-meter probe (model EMP-411, Electromagnetic Probe Company, lumen size, 11-mm circumference) was placed around the exposed renal artery and renal blood flow was monitored with a square-wave electromagnetic flow meter (model *321*, Carolina Medical Electronics). The flow meter was set to record mean blood flow and was adjusted to zero flow by briefly occluding the renal artery distal to the flow-meter probe. Blood pressure was monitored from the carotid artery. Both blood flow and blood pressure were recorded on an Offner recorder. A solution containing 0.9% NaCl, 0.4% inulin, and 0.08% *p*-aminohippurate (PAH) was infused into the right femoral vein at a rate of 0.25 ml/kg/min for at least ½ hour before and throughout the entire experiment.

WY-5256 was administered intravenously as a suspension in saline. A priming dose of 0.25 mg/kg plus an infusion of 0.25 mg/kg/hour or a priming dose of 2.5 mg/kg plus an infusion of 2.5 mg/kg/hour were given.

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