

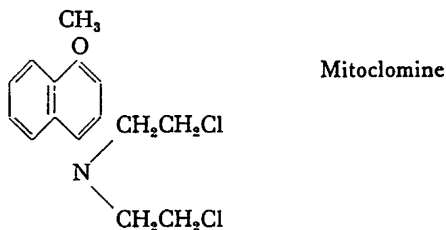
# Investigations into the Lymphopaenic and Immunosuppressive Properties of the Antitumour Agent, Mitocloimine

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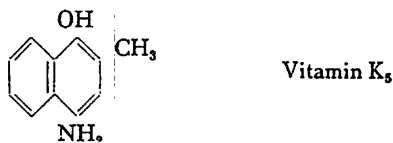
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The formula of Mitocloimine (W.B. 7007) was suggested by *Granger* in 1966 (1) as that of a useful cytotoxic agent, and confirmed in a number of experimental tumours.



It had been shown previously that the synthetic vitamin K, Synkavit was concentrated by some tumour systems (2, 3) and possessed useful radiosensitising properties (4, 5). Vitamin K<sub>5</sub>, although not studied to the same extent as Synkavit, was also thought to share those properties and the molecule lent itself to substitution of the amine radicle with a N-N-bischloroethyl group.



Previous studies by *Whisson* and *Connors* (6, 7) had demonstrated that in aniline mustard compounds the group in the para position to a mustard on the benzene ring could influence the overall toxicity and cytotoxic activity of the compound. A para-hydroxy aniline mustard derivate had increased toxicity and decreased therapeutic index when compared with aniline mustard itself, but a paramethoxy derivative showed an increased cytotoxic activity with decreased toxicity. The vitamin K<sub>5</sub> molecule was, therefore, modified further by substituting a methoxy for a hydroxy group. *Granger* (1) reported that this compound possessed useful cytotoxic activity against various animal tumour systems. An additional observation was that of a concurrent selective depressant effect on the blood lymphocyte without reduction of the polymorphonuclear count.

The present experiments were designed to investigate the lymphopaenic effect more fully, its relation to dosage, frequency and site of administration, side effects and maximum tolerated amounts. The mouse was used as the experimental animal. The effects on haemagglutinin formation and skin allograft rejection were employed as measures of possible immunosuppressive properties.

### *Materials and Methods*

*Animals.* Pathogen free adult albino mice weighing between 20 and 28 grammes were obtained from the Imperial Chemical Industries Laboratories, Macclesfield, England, and used in the first part of the experiment for toxicity and systemic effect studies. CBA strain adult mice were employed as recipients for skin grafts and A strain animals as donors. Dog erythrocytes were used for immunisation and haemagglutination studies. Blood was taken from one animal only throughout the experiments.

*Mitocloimine* was dissolved in sterile warmed Arachis oil in a concentration of 10 mgs per 1 ml oil in all the experiments apart from the toxicity studies when higher concentrations were required for larger quantities. The drug is insoluble in aqueous diluents. No more than 1 ml of the solution was given intraperitoneally or orally in one dose. Oral administration was by means of a narrow polyethylene cannula passed into the stomach.

*Blood counts and histology.* Blood was obtained by tail vein bleeding. Haematocrit values were estimated by the micropipette method. Blood and bone marrow smears were stained with Leishman stain. Mice were killed at varying intervals following single and repeated drug doses. Tissues were fixed in 10% formal-saline solution and stained with haematoxylin and eosin, with methyl green-pyronin and Perl's stain for haemosiderin in selected cases. Inguinal and mesenteric lymph nodes, spleen, thymus, liver, kidneys, ileum and lungs were examined histologically.

*Red cell immunisation and haemagglutination.* Heparinised dog blood was washed three times in 0.9 per cent saline, the buffy coat was removed and a final suspension of 400 million cells per ml was made. Two hundred million cells were injected into the tail vein of the mouse. Haemagglutination tests were performed in 0.9 per cent saline suspension using Takatsi microtitre equipment on days 12, 14, 18 and 25. On the 25th day a second dose of red cells was given and the secondary response was measured on the 7th and 12th days thereafter.

*Skin grafting* was performed by the method of *Billingham and Medawar* (8). Dressings were removed and grafts were first inspected on the 12th day. The point of rejection taken was when the graft became uniformly firm and bloodless.— Results were charted in averages and ranges given where applicable. — A total of 199 mice was used.

### *Results*

Following single intraperitoneal injection of mitocloimine, 400 mg per kg body weight, all the 12 animals in the group died within 12 days (Fig. 1). Progressive weight loss occurred from the first day. The blood lymphocyte count was reduced as early as the first day and continued to fall rapidly to below 500 per cu. ml on the 7th day. The polymorphonuclear count was increased but the total white cell count remained little changed throughout the period of observation.

Histological sections were examined on the 6th, 7th and 11th days. On the 6th day the thymus showed advanced lymphoid depletion of the cortex. On the 7th day the changes were more severe with the medulla also affected. On the 11th day the thymic structure was completely destroyed. The effects on the spleen were more irregular. On the 6th day severe lymphoid depletion was present. Similar but less severe changes were seen on the 7th and 11th days with retention of follicular structures. Each of the spleens showed extensive accumulation of haemosiderin. No abnormality was found in the liver, heart, kidneys, lungs or ileum.

Fig. 1 Effect of intraperitoneal Mitocloimine, 400 mg per kg. (Points represent averages, vertical lines ranges, the arrow the day of Mitocloimine injection.)

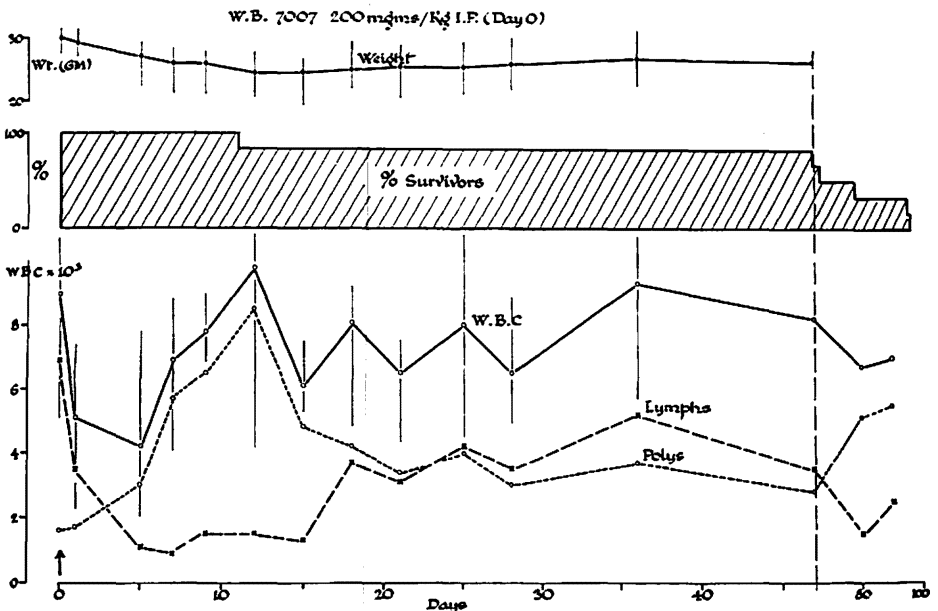
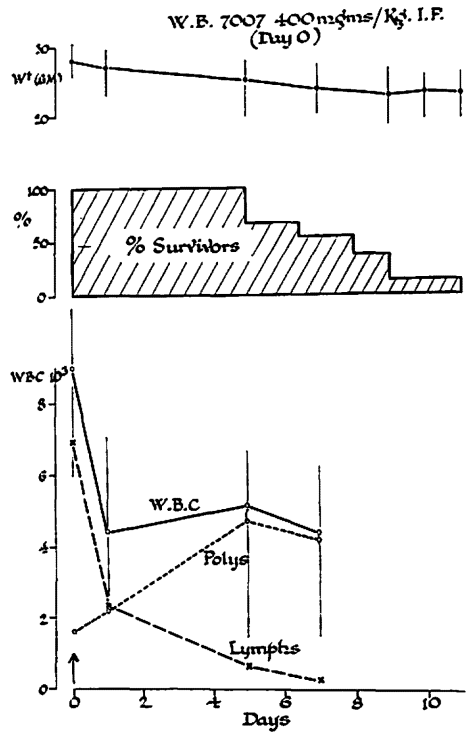


Fig. 2 Effect of intraperitoneal Mitocloimine, 200 mg per kg. (Symbols as in Fig. 1.)

Twelve animals were injected intraperitoneally with a single dose of 200 mg per kg body weight. Survival, weight and leucocyte count changes are shown in Fig. 2. The blood lymphocyte count was reduced from the 2nd day and remained in the region of 1,000 to 1,500 per cu. ml from the 5th to the 15th days and then rose gradually after-

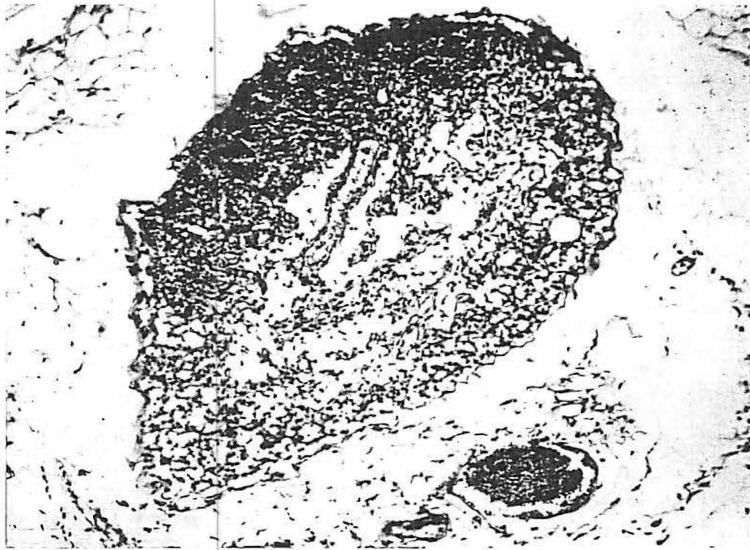


Fig. 3 a Mouse inguinal lymph node 11 days after I. P. Mitoclomine, 200 mg per kg. (H & E  $\times$  80.)

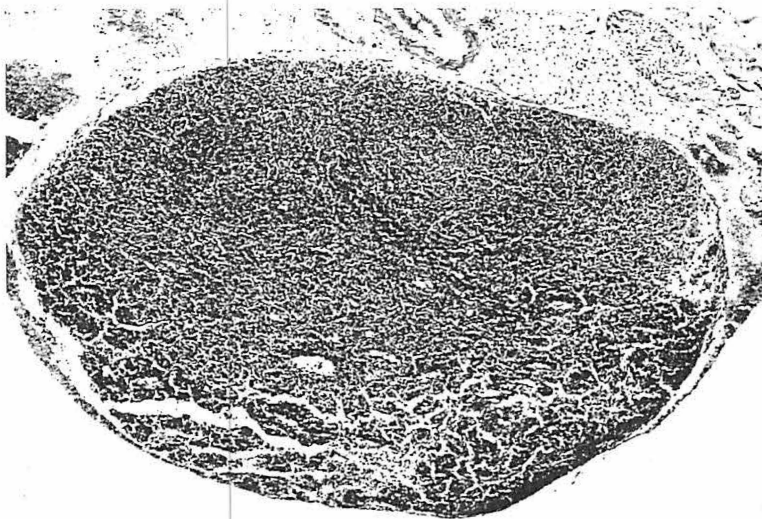


Fig. 3 b Inguinal lymph node from control mouse.

wards. Polymorphonuclear leucocytosis accompanied the period of maximum lymphoid depression when weight loss was also most marked. The animals then gained weight and appeared to stay well after the death of two on the 11th day. Lymphopaenia began to be marked once again on the 47th day and increased to the time of death of all the animals on the 96th day.

Tissues were examined at intervals from 1 to 96 days after injection. On the 1st day the lymphoid tissues were normal but thereafter the sequence of changes in the spleen, thymus and lymph nodes was slightly different. On the 4th and 11th days the lymph nodes showed severe lymphoid depletion (Fig. 3) but the changes were less marked thereafter. From the 4th to the 35th day the spleen showed moderate but varying degrees of lymphoid depletion and haemosiderin accumulation, mostly affecting the peripheral pulp. However, on the 50th and 96th days a striking change had occurred with almost complete atrophy of the splenic lymphoid elements (Fig. 4). The thymus showed slower

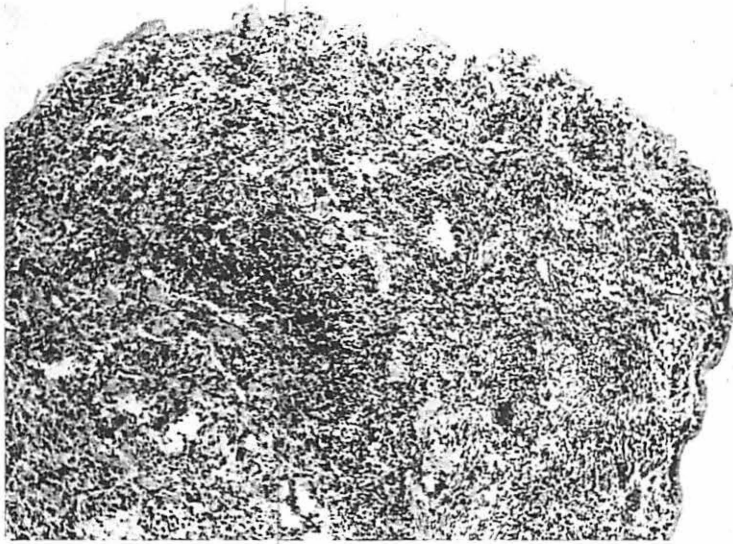


Fig. 4 a Mouse spleen 50 days after I.P. Mitocloquine, 200 mg per kg. (H & E  $\times$  80.)

but progressive changes, being normal on the 7th and exhibiting moderate to severe cortical lymphoid depletion on the 11th day. By the 24th and 35th days this process involved the medulla also. On the 50th and 96th days the thymus could not be identified on dissection.

The effect of repeated intraperitoneal injections in 12 mice is shown in Fig. 5 when 100 mg. per kg was given twice weekly for three doses, reducing to 50 mg per kg. Weight loss was progressive and all the animals died by the 38th day. Severe lymphopaenia occurred and total lymphocyte counts were 500 per cu. ml or lower towards the end of the investigation. A polymorphonuclear response was noted initially as in the previous groups, but the count then returned to normal.

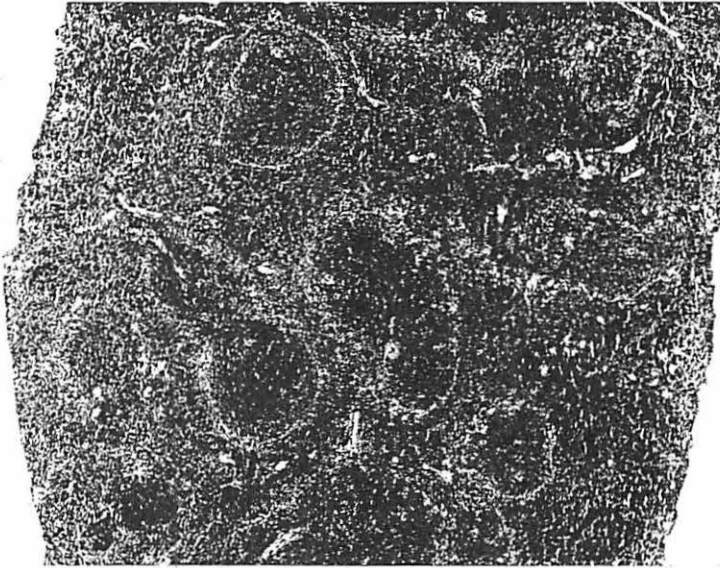


Fig. 4 b Spleen from control mouse.

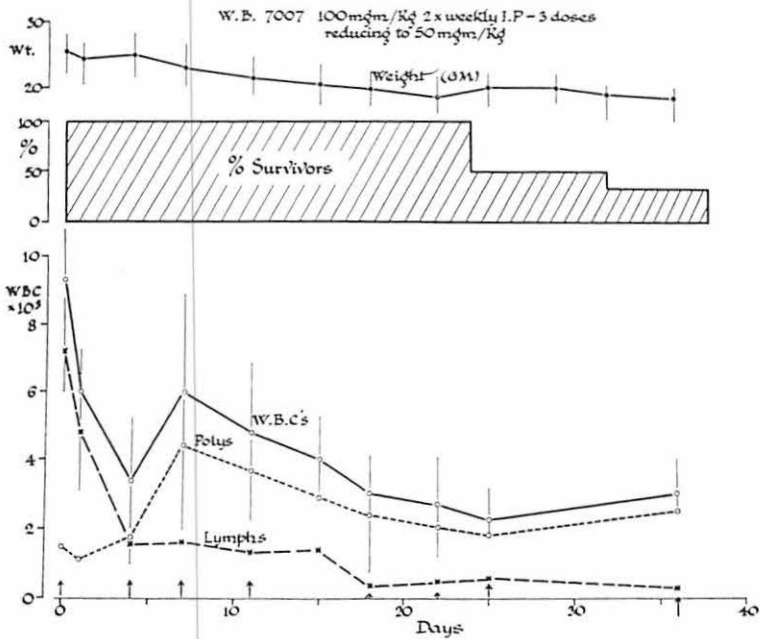


Fig. 5 Effect of repeated intraperitoneal Mitoclozine. (Symbols as in Fig. 1.)

Histological examination of animals dying between the 24th and 35th days showed both the cortical and medullary elements of the thymus to be severely atrophied. Lymph nodes were less affected. Spleens showed moderate lymphoid depletion of the inter-follicular pulp with the follicular elements remaining.

Four mice were given single doses of Arachis oil intraperitoneally to exclude a possible effect by the carrier. Two received 0.5 ml and two 1 ml. Over a four week period of observation the animals remained well, maintained their weight, lymphocyte and polymorphonuclear counts were unchanged.

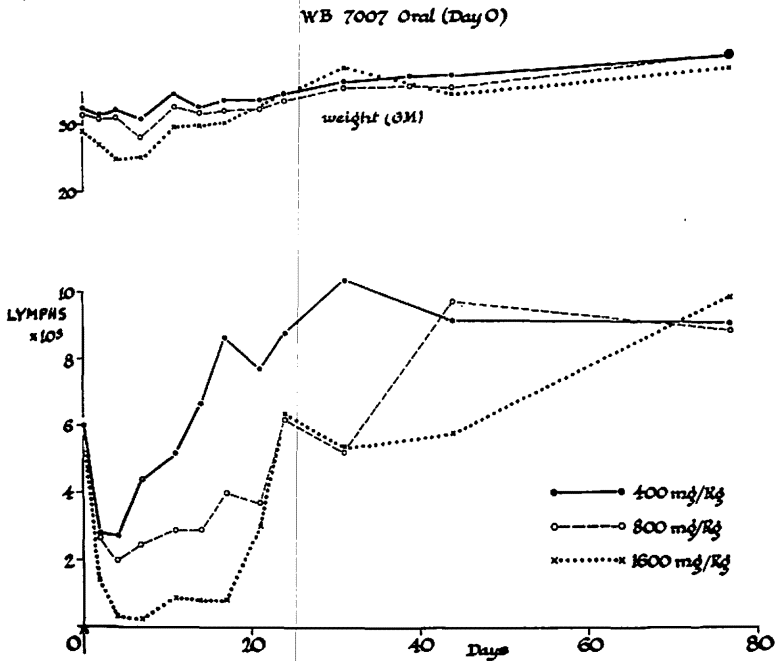


Fig. 6 Effect of oral Mitocloimine.

Single doses of Mitocloimine were given orally, 400, 800 and 1600 mg per kg body weight to groups of 8 mice. Results are shown in Fig. 6. Lymphopaenia occurred with all 3 doses. The onset was later than that following intraperitoneal administration, but the depression was marked particularly with the two larger quantities in which it also lasted longer. Mortality rates were not assessed because some of the animals were sacrificed early for histological examination, but it was higher than 50 per cent in the 800 and 1600 mg groups. Normal counts were reached in 2 weeks with the 400 mg per kg dose and in 3 weeks after 800 and 1600 mg per kg. Animals which survived this time then remained well over a 7 months period of observation. Histological examination of the lymphoid tissues and thymus at the end of that period showed no abnormality. Weight loss and polymorphonuclear leucocytosis once again accompanied the lymphopaenia and the effects were related to the dosage. Following administration of 400 mg per kg marked central lymphoid depletion was seen on the 4th day and at that stage the

changes were most severe. The lymph nodes showed atrophy, the thymus depletion of cortical lymphocytes (Fig. 7) and the spleen early lymphoid depletion of the interfollicular pulp. The plasma cell population appeared normal. On the 6th and 14th days

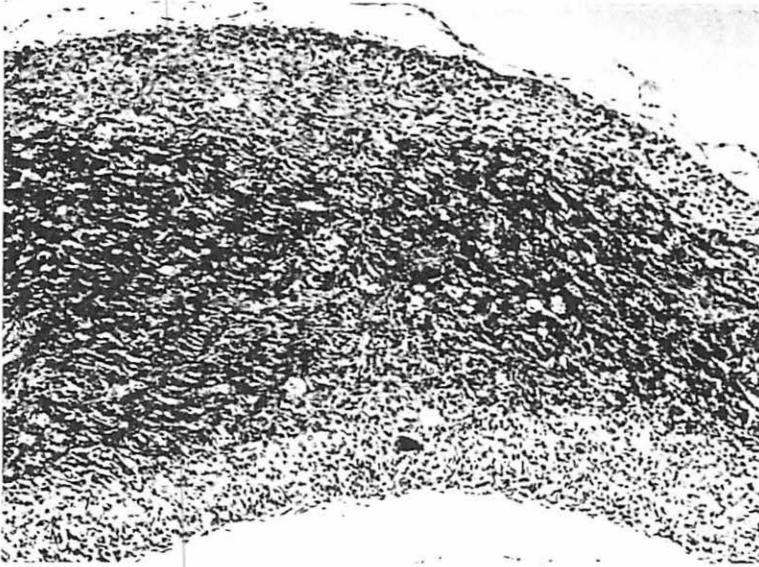


Fig. 7 a Mouse thymus 4 days after oral Mitocloimine, 400 mg per kg. (H & E  $\times 100$ .)

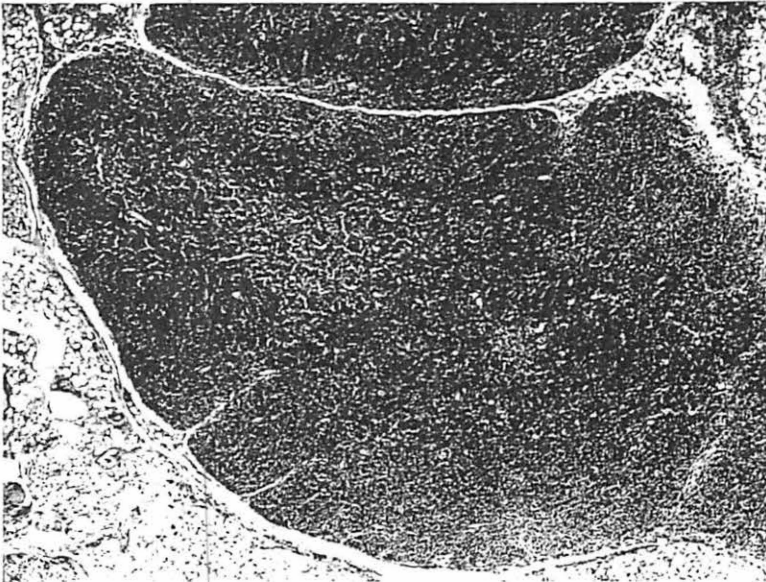


Fig. 7 b Thymus from control adult mouse.



the depletion in the lymphoid tissues was less marked but both the spleen and lymph node contained significantly increased numbers of plasma cells. By one month the spleen had returned to normal but again showed increased groups of plasma cells. At 7 months the lymphoid tissues appeared completely normal. After 800 mg per kg the spleens showed more severe lymphoid depletion on the 2nd day than with the 400 mg dose although many follicles still remained. The changes were less severe on the 6th day when haemosiderin deposition and prominent groups of plasma cells were seen. Only minimal lesions were present on the 14th day and from the end of the first to the 7th months the spleens were normal apart from some increase in plasma cells in the first 4 weeks. On the 6th day the thymus showed severe cortical atrophy but was normal on day 14. By day 14 depletion in the lymph nodes was decreasing. Once again an increase in plasma cells was noted, up to one month. The changes were very much more severe following 1600 mg per kg. At day 7 the spleen showed almost total lymphoid depletion. In animals which survived the initial lymphoid depletion, complete lymphoid recovery occurred.

Twelve mice received oral Mitoclomine, 200 mg per kg twice weekly. Results are shown in Fig. 8. Lymphopaenia was maintained from the end of the first week. Weight loss did not occur. After two deaths on the 6th day there was no further mortality till the 65th day, and on the 76th day two thirds of the animals had died. Histological examination was not performed in this group.

Bone marrow smears were examined in 11 animals receiving varying doses of Mitoclomine, differential counts were made and compared with those of controls. Results are set out in Table 1.

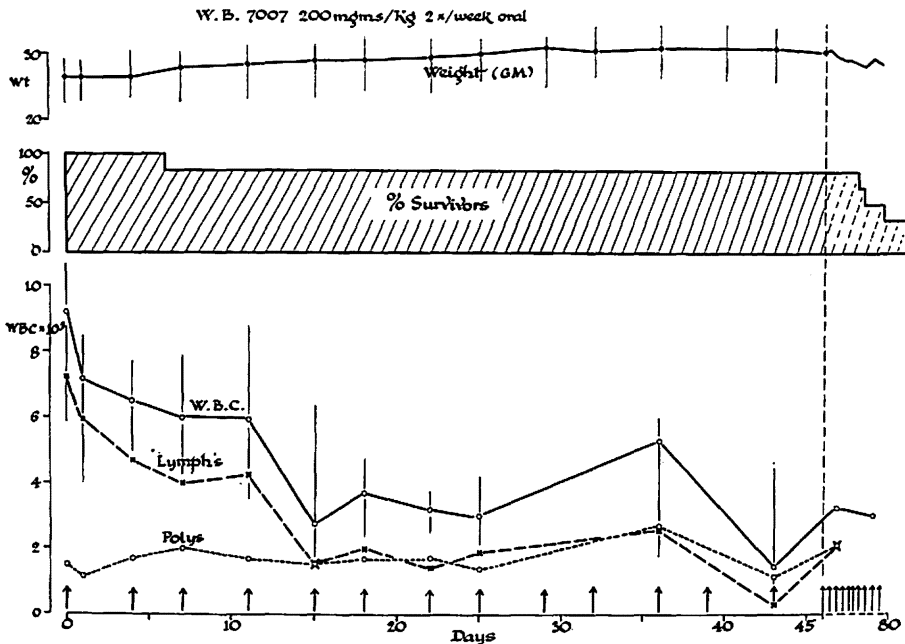


Fig. 8 Effect of repeated oral Mitoclomine. (Symbols as in Fig. 1.)

Table 1 Bone Marrow Changes Following I. P. Mitocloimine in Single and Repeated Doses.

	Control	Percentage counts										
		200 mg/kg					400 mg/kg			800 mg/kg	100 mg/kg 2× weekly	
Days	-	4	11	23	35	61	6	7	11	7	25	36
Reticulum cell	0.5	1	0.5	1	1	2.5	-	-	-	0	0.5	1
Myeloblast	2	4	1.5	1	3.5	1.5	1	5	3	2.5	3.5	4
Premyelocyte	2	2	1.5	2	1	3	1.5	5	2	2	2	3
Myelocyte N	2	2	2	3	3.5	5.5	4	4	4	3.5	3.5	8
E	0.5	-	1	-	-	0.5	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-
Meta -												
Myelocyte N	30	40	39	26	33	50	55	62	38	49	22.5	34
E	1	-	1	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-
Mature												
Polymorph N	2.5	30	29	16	16	6.5	10.5	17	36	11	3	4
E	-	-	1	1.5	-	1.5	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-
Erythroblast	5	2	2.5	1.5	2	1.5	3	0.5	2	4.5	2	1
Early Normobl.	14	2	3	8	7	1.5	5	0.5	1	3.5	5	5
Late Normoblast	30	11.5	5.5	20	19	14	11	1	1.5	9	48	28
Megakaryocyte	0.5	1	1	0.5	1	1	0.51	1	1	1	1	1
Lymphocyte	10	3	10	18	10	10	7	3	10	12	8	9
Monocyte	-	1.5	1.5	1.5	3	1	1.5	1	1.5	2	1	2

Following single intraperitoneal injection of 200 mg per kg the early forms of white cells showed little change but there was a striking increase in the proportion of mature polymorphs at day 4, returning to almost normal on day 61. The number of lymphocytes was initially reduced with recovery afterwards. The depression of erythropoiesis was probably not absolute but was related to the increase in polymorphs. Similar changes were seen after the 400 and 800 mg per kg doses. Recovery had not occurred on day 11 after which no further readings were obtained. The lymphocytes were not affected in the latter group. A marked increase in the proportion of normoblasts was seen on days 25 and 36 after repeated intraperitoneal injections. In view of subsequent findings of excessive haemosiderin deposition the most likely cause was that of a haemolytic process.

Haemosiderin deposition of considerable degree was noted in the spleen in most cases. The results are expressed in Table 2 as ranging from nil to ++++ according to severity. After a single intraperitoneal dose, haemosiderin appeared in the spleen during the first week, rose to a maximum by day 4 and then decreased over the following month. The amount increased once again when later deterioration occurred in mice that had been given 200 mg per kg. With oral dosage, the accumulation was dose related both as to density and rapidity of accumulation.

Table 2 Haemosiderin Deposition in the Spleen.

400 mg/kg		Single oral dose 800 mg/kg		1,600 mg/kg	
Day	4 0	Day	2 0	Day	7 ++++
	6 +		6 +++		
	14 +		14 +++		
	30 ++		30 ++		
	300 ++		300 +		

200 mg/kg		Single I.P. Dose 400 mg/kg		800 mg/kg	
Day	1 +	Day	6 ++++	Day	6 ++++
	4 ++++		7 ++++		
	7 ++++		11 ++++		
	11 ++++				
	14 +++				
	24 ++				
	35 +				
	50 ++				
	96 ++				(Day 0 = 0)

Table 3 Haemagglutinin Titres.

	Days	R.B.C.'s only	Mitoclomine 200 mg/kg i.p. + R.B.C.'s same day	Mitoclomine 200 mg/kg i.p. + R.B.C.'s 3 days previously
Primary	12	1:4 (0-16)	1:2 (0-4)	1:6 (0-32)
Response	14	1:8 (2-64)	1:3 (0-16)	1:6 (0-128)
	20	1:4 (2-16)	1:4 (0-32)	1:8 (0-512)
	25	1:2 (0-8)	1:2 (0-4)	1:8 (2-32)
Secondary	7	1:256 (32-2048)	1:256 (16-2048)	1:256 (64-1024)
Response	12	1:2048 (512-8192)	1:2-48 (64-65176)	1:256 (32-4096)

Figures in brackets = range.

Results of haemagglutination studies in three groups of 12 mice are shown in Table 3. The first group was that of controls which received an immunising injection of dog red cells and a boosting dose on day 25. Mitoclomine 200 mg per kg body weight intraperitoneally was given simultaneously with the red cells on days 0 and 25 in the second group. In the third group the drug was administered 3 days prior to the red cells.

Haemagglutination titres during the primary or secondary response were not significantly altered from those of controls when Mitocloimine was given at the same time or before the antigenic stimulus.

Skin allografts were uniformly rejected in all animals when examined on the 12th day following a single intraperitoneal injection, 200 mg per kg, given on the day of grafting. In control animals graft rejection is complete by the 12th day (range 9–12 days).

### *Discussion*

Mitocloimine was found to have a marked depressant effect on the lymphoid tissues in the mouse. The lymph nodes, spleen and thymus and the circulating blood lymphocytes were affected. The action was confined essentially to the lymphocyte and its precursors. The plasma cell population was not depressed and in some cases it appeared to be increased. The polymorphonuclear count was initially increased but subsequently it settled to normal. Repeated and larger doses eventually reduced the peripheral polymorphonuclear count. A red blood cell destructive effect was suggested by a relative increase in the bone marrow normoblast population accompanied by increased haemosiderin deposition in the spleen.

Higher dosage was tolerated better orally than intraperitoneally. Mortality, toxicity and lymphoid depression were less with oral administration and recovery was faster and appeared complete in those animals which survived the initial period of lymphoid depression. A single oral dose in the range given produced maximal lymphoid depression from the third to the 14th days, which rapidly decreased afterwards. After intraperitoneal administration recovery was slower. Lymphoid depression was still apparent at 4 weeks and subsequently increased with the death of all the animals following a single dose of 200 mg per kg body weight 3 months after injection. The cause for the difference of effect between oral and intraperitoneal administration was not investigated. Possibilities include partial absorption in the alimentary canal, destruction or detoxication of the orally administered Mitocloimine. Prolonged, moderate lymphoid depletion could be achieved by repeated oral administration of the drug.

In view of the severity of the lymphoid depletion the possibility of an immunosuppressive affect was considered. Both humoral and delayed hypersensitivity-type responses were investigated using the ability to form haemagglutinins and the rejection of skin allografts as parameters. Maximum tolerated doses of Mitocloimine were given over the relevant period of observation. The development of haemagglutinin titres was unaffected when the drug was given 3 days before and at the same time as the antigenic stimulus. These findings are at variance with those of *Vahlensieck* (9) who used the rat as the experimental animal but in agreement with results obtained by *Santos* (10) who noted an actual increase in haemagglutinin titres in the rat following Mitocloimine administration. It is possible that repeated dosage at a lower level particularly when given orally may have a different effect, and this is being tried.

The life of skin grafts was not prolonged though severe lymphoid depletion was produced. In view of the immunosuppressive properties of some other nitrogen mustard compounds (11) the results were a little disappointing. The immunity of the plasma cell

to the action of the drug probably provides the answer and the number of these cells appeared to be increased in some cases. It is possible that a useful effect could be obtained in combination with immunosuppressive agents with a different mode of action.

### Summary

Mitocloimine (W. B. 7007) introduced as an antitumour agent is a Vitamin K derivative with a nitrogen mustard group incorporated in the molecule. It has a marked depressant effect on both the fixed and the peripheral lymphoid tissues. The relation of this effect to dosage, toxicity, frequency and site of administration was investigated in the mouse. In view of this lymphopaenic action, its use as an immunosuppressive agent was considered. When employed alone, there was no effect on the soluble or delayed-type responses, probably because of its plasma cell sparing response action which was found. However, by virtue of its almost specific lymphopaenic properties it may be useful in immuno-therapy if combined with other standard agents.

### Acknowledgments

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