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FURTHER OBSERVATIONS ON THE EFFECT OF AMINOPTERIN, A-METHOPTERIN AND *CITROVORUM* FACTOR ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

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A number of workers have reported on the effect of aminopterin and A-methopterin on the course of the growth of the Rous sarcoma and certain transmissible tumours in chicks. Woll (1948) and Little, Sampath, Paganelli, Locke and Subbarow (1948) have shown that a number of folic acid antagonists completely or partly inhibit the growth of the Rous chicken sarcoma although some toxic effects were encountered. The latter workers showed that the toxicity of aminopterin, in particular, could be partially neutralized by either pteroyldi- or pteroyltriglutamic acid in a 25 to 1 ratio, but such mixture showed no virus-inhibiting effect. Stock, Biesele, Burchenal, Karnofsky, Moore and Sugiura (1950) have also demonstrated that several analogues of folic acid exert an adverse effect upon tumour tissue. Ringsted (1952) fed a balanced chick mash containing aminopterin (5 mg./kg. of food) and found that the survival rate of chicks inoculated with Rous sarcoma was significantly higher at the end of the first week compared with that of chicks receiving no drug. The mean time of first appearance of the tumour in the survivors was also greater in the chicks receiving aminopterin, but the tumour incidence in both the medicated and control groups was 100%. Bessis and Freixa (1950) found that aminopterin and A-methopterin offered partial protection against a transmissible erythroblastosis in young chicks, but some intoxication was encountered. Ringsted (1953) showed that aminopterin in a chick mash at levels of 5, 10, 20 and 40 mg./kg. increased the mean induction time of a Mill Hill endothelioma in young chicks. The two higher levels proved toxic, and the drug at all levels had no effect on the total number of takes and subsequent growth-rate of the tumours. Darcel (1953a) failed to show any increase in the time taken for tumours to appear when aminopterin or A-methopterin were given orally to chicks bearing tumour strain RPL 12. The drugs were administered directly into the crop in aqueous suspensions (0.1 and 1.0 mg. of each drug/day). No toxic symptoms were reported.

Recently, *citrovorum* factor, a substance closely related to folic acid, has been found to be many times more effective than folic acid itself in reversing the toxicity of folic acid antagonists in the rat, chick, and man, and in this paper a number of observations are presented on the effect of aminopterin, A-methopterin and synthetic *citrovorum* factor on the growth of transplantable lymphoid tumour strains isolated from cases of avian leukosis.

METHODS

The general characteristics of the tumour strains used in the present study, RPL 12 and 19, have been described by Darcel (1953b). A tumour develops at the site of inoculation followed by rapid metastasis, in the absence of leukaemia, to the viscera, especially the liver.

Crossbred (B.R. × Br.L. and B.R. × W.L.) chicks were inoculated with tumour cell suspensions either at two or seven days of age. The birds were maintained in small metal cages and fed freely on a balanced chick mash.

The tumour cell suspensions were prepared in physiological saline with a special mincer (Craigie, 1949) from liver in which tumours were growing (artificially inoculated). The tumour suspension (0.25 ml.) was inoculated subcutaneously above the pectoral muscle. Concentrations of the tumour mince corresponded to 1/10th of the original tumour in all experiments. The chicks were palpated daily in order to calculate the mean time of first appearance of tumours in individual groups. The mean survival times of the experimental groups were also determined.

The drugs were administered either intraperitoneally or in the food. The *citrovorum* factor (20 μg.) was given intraperitoneally each day.

TABLE I

THE EFFECT OF INTRAPERITONEAL ADMINISTRATION OF AMINOPTERIN AND *CITROVORUM* FACTOR ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

Expt. No.	Tumour	Chicks Used	Age (Days)	No. Inoculated	Amount of Aminopterin Administered (μg. Daily)	*Citrovorum* Factor (μg. Daily)	Tumours		Deaths	
							Tumours Palpated	Mean Time of First Appearance (Days) ± Mean Deviation	No.	Survival Time (Days) ± Mean Deviation
1	RPL 19	BR/Br.L × BR	2	10	Nil	Nil	4	5·00±0	10	6·40±0·92
	,,	,,	2	10	100	,,	0	—	10	3·80±1·20
	,,	,,	2	10	100	20	5	6·80±1·04	9	7·44±1·16
2	RPL 12	BR × Br.L	2	10	Nil	Nil	10	7·80±0·80	10	13·20±1·04
	,,	,,	2	10	50	,,	0	—	10	3·60±0·72
	,,	,,	2	10	50	20	8	7·88±0·88	9	11·50±0·50
4	RPL 19	BR × W.L.	7	10	Nil	Nil	10	6·40±0·48	7	*10·00±0·56
	,,	,,	7	10	50	,,	0	—	10	7·50±2·60

* Three chicks were killed on the 25th day after inoculation and in each only a small regressing pectoral tumour was found.

RESULTS

In general, both aminopterin and A-methopterin were found to be toxic for young chickens, although a considerable variation in susceptibility was encountered. Aminopterin, in particular, proved extremely toxic when administered intraperitoneally to two-day-old chicks in daily doses of 50 or 100 μg. All the birds died within four days of the commencement of therapy and it was impossible to ascertain if any tumour-inhibiting action had occurred (Table I). These birds rapidly lost weight, anaemia and leucopenia were observed, and on post-mortem examination the liver was slightly enlarged and intensely yellow—probably the result of an accumulation of unmetabolized aminopterin. *Citrovorum* factor gave full protection against this toxicity when administered intraperitoneally at 20 μg./day. On the other hand, any possible tumour-inhibiting effect by the aminopterin was neutralized, there being no significant difference between the number of tumours palpated, the mean times of their first appearance, or mean survival times in either the treated or control groups (Table I, Expts. 1 and 2). The administration of aminopterin intraperitoneally to older chicks (50 μg. daily) whilst apparently preventing the appearance of palpable tumours ultimately caused a 100% mortality from intoxication (Table I, Expt. 4). The same picture was seen when aminopterin was administered in the diet (10 mg./kg. of food), although in week-old chicks it appeared to be less toxic (Table II, Expts. 4 and 5). No palpable tumours could be detected in these birds, with the exception of one in Expt. 5, and their mean survival time was significantly prolonged. With the exception of three survivors

TABLE II

THE EFFECT OF THE DIETARY ADMINISTRATION OF AMINOPTERIN ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

Expt. No.	Tumour	Chicks Used	Age (Days)	No. Inoculated	Dietary Level of Aminopterin (mg./kg.)	Tumours		Deaths	
						Tumours Palpated	Mean Time of First Appearance (Days)	No.	Survival Time (Days)
3	RPL 19	BR × W.L.	2	10	Nil	10	6·00±0	10	7·90±0·18
	,,	,,	2	10	10	0	—	10	5·10±1·14
4	RPL 19	BR × W.L.	7	10	Nil	10	6·40±0·48	10	10·00±0·56
	,,	,,	7	10	10	0	—	10	13·56±2·05[1]
5	RPL 12	BR × Br.L.	7	10	Nil	8	6·00±0	8	11·00±0[2]
	,,	,,	7	10	10	1	10·00±0	10	24·50±3·90[3]
6	RPL 19	BR × Br.L.	7	10	Nil	10	6·70±0·42	9	13·56±3·65
	,,	,,	7	10	10	0	—	10	3·43±0·65[4]

NOTES.—[1] One chick died from intoxication after 4 days and has not been included in the calculation of the mean survival time.
[2] Two chicks were killed after 35 days and no evidence of tumours was found.
[3] Four chicks died from intoxication after 4.9 days and have not been included in the calculation of the mean survival time.
[4] Three chicks survived to the 13th day and no tumours were observed.

TABLE III

THE EFFECT OF INTRAPERITONEAL ADMINISTRATION OF A-METHOPTERIN AND *CITROVORUM* FACTOR ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

Expt. No.	Tumour	Chicks Used	Age (Days)	No. Inoculated	Amount of A-methopterin Administered (μg. Daily)	*Citrovorum* Factor (μg. Daily)	Tumours		Deaths	
							Tumours Palpated	Mean Time of First Appearance (Days)	No.	Survival Time (Days)
1	RPL 19	BR × BR.L × BR	2	10	Nil	Nil	4	5·00±0	10	6·40±0·92
	,,	,,	2	10	100	,,	0	—	10	7·90±1·16
	,,	,,	2	10	100	20	8	5·00±0	10	6·10±0·72
2	RPL 12	BR × BR.L	2	10	Nil	Nil	10	7·80±0·80	10	13·20±1·04
	,,	,,	2	10	100	,,	0	—	10	*3·56±0·70
	,,	,,	2	10	100	20	10	7·00±0	10	11·10±0·36
3	RPL 19	BR × W.L.	2	10	Nil	Nil	10	6·00±0	10	7·90±0·18
	,,	,,	2	10	50	,,	4	7·75±0·38	10	10·00±0·40
4	RPL 19	7BR × W.L.	7	10	Nil	Nil	10	6·40±0·48	7	10·00±0·56
	,,	,,	7	10	50	,,	0	—	10	7·70±3·44

* One chick survived for 14 days before dying from liver lesions. This was not included in the calculations of the mean survival time.

in Expt. 6, all the birds eventually succumbed and were found to have diffuse lesions in the liver as the result of a lymphocytic infiltration.

A-methopterin, in general, proved somewhat less toxic than aminopterin, but once again considerable variation in susceptibility was encountered. In birds which were resistant to intoxication, both the intraperitoneal and dietary administration of the drug considerably reduced the number of palpable tumours and increased their time of first appearance (Tables III and IV). It was found, however, that A-methopterin in the diet at 10 mg./kg. had no effect on the number of tumours palpated, the mean time of their first appearance or survival time in two-day-old birds bearing RPL 19 (Table IV, Expt. 3). When the dose of the drug was raised to 20 mg./kg. and given to slightly older birds there was a marked reduction in the numbers of tumours palpated and an increase in survival time. In a single expt. with RPL 12, A-methopterin at 10 mg./kg. of diet showed some tumour-inhibiting effect (Table IV, Expt. 5). As with aminopterin, full protection against any toxicity was given by *citrovorum* factor (20 μg. daily intraperitoneally), but any tumour-inhibiting action was neutralized.

DISCUSSION

From the results presented, there is some evidence that both aminopterin and A-methopterin can influence the growth of transplantable avian lymphoid tumours. The results, however, are complicated by the toxicity of these two folic acid antagonists, although a wide variation in susceptibility has been encountered. Bennette (1952), in experiments with A-methopterin in mice, concluded that the association between the toxicity and tumour growth inhibition was so close that no confidence could be placed in the selectivity of the drug against certain sarcomata. Our results, in

TABLE IV

THE EFFECT OF THE DIETARY ADMINISTRATION OF A-METHOPTERIN ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

Expt. No.	Tumour	Chicks Used	Age (Days)	No. Inoculated	Dietary Level of A-methopterin (mg./kg.)	Tumours		Death	
						Tumours Palpated	Mean Time of First Appearance (Days)	No.	Survival Time (Days)
3	RPL 19	BR × W.L.	2	10	Nil	10	6·00±0	10	7·90±0·18
	,,	,,	2	10	10	10	6·00±0	10	8·20±0·36
4	RPL 19	BR × W.L.	7	10	Nil	10	6·40±0·48	10	10·00±0·56
	,,	,,	7	10	20	0	—	9	13·89±1·46
5	RPL 12	BR × Br.L.	7	10	Nil	8	6·00±0	8	11·00±0[1]
	,,	,,	7	10	10	2	8·00±1·00	9	13·83±3·44[2]
6	RPL 19	BR × Br.L.	7	10	Nil	10	6·70±0·42	9	13·56±3·63
	,,	,,	7	10	20	0	—	10	15·50±1·33[3]

NOTES.—[1] Two chicks were killed after 35 days and no evidence of tumours was found.
[2] Three chicks died from intoxication after 4.7 days and have not been included in the calculation of the mean survival time.
[3] Four chicks died from intoxication after 4.9 days and have not been included in the calculation of the mean survival time.

part, tend to confirm this conclusion—especially when the drugs are administered intraperitoneally to very young chicks. A greater resistance to intoxication was found when the drugs were administered in the food to week-old birds. In such resistant birds there was a considerable reduction in the number of palpable tumours and an increase in the time taken for them to appear, irrespective of the tumour strain used. In addition, the survival time was increased. However, when death intervened a post-mortem examination revealed that in the majority (even in the absence of a pectoral tumour) metastasis to the viscera had occurred, and especially to the liver.

The toxic effects of these two drugs could be overcome by injections of synthetic *citrovorum* factor, as has recently been demonstrated in the rat and chick (Sauberlich, 1953 ; Sauberlich and Schaefer, 1954). At the same time, this *citrovorum* factor blocked any tumour-inhibiting action. Because of the small amount of *citrovorum* factor available it was not possible to determine the minimum concentration which could prevent this intoxication, and yet permit the drugs to exert any possible tumour-inhibiting action. For the same reason, it was not possible to investigate the effect of its oral administration.

Darcel (1953a) found that the oral administration of suspensions of either aminopterin or A-methopterin failed to influence the growth of tumour strain RPL 12 and he found no evidence of intoxication. This suggests that little or no absorption of either drug had occurred. The toxic effects encountered in our experiments were poor growth, anaemia and leucopenia, and death. Bessis and Freixa (1950) administered the two antagonists intramuscularly in much higher doses (0.5 mg. of aminopterin and 4 mg. of A-methopterin) and reported only a small number of chicks showing intoxication. This route of administration may have permitted the very slow dispersion of the drug from a high local concentration, so that the tumour-inhibiting action was exerted without drastic interference with folic acid or *citrovorum* factor enzyme systems.

The above results suggest that the effect of these drugs on the growth of lymphoid tumours should be extended, especially as their toxic effect may be controlled by *citrovorum* factor. On the other hand, although there is some evidence that inhibition of tumour growth occurs in those chicks resistant to intoxication, death ultimately ensues from a diffuse lymphocytic infiltration of the viscera.

Summary

1. Aminopterin (4-aminopteroylglutamic acid) given intraperitoneally to chicks, bearing either tumour strain RPL 12 or RPL 19, in doses of 50 or 100 μg./day was highly toxic, and resulted in poor growth, anaemia, leucopenia and death. For this reason it was not possible to ascertain if any tumour inhibition had occurred.

This toxicity could be counteracted by *citrovorum* factor administered intraperitoneally, but any tumour-inhibiting action was neutralized.

2. Aminopterin (10 mg./kg. of diet) was somewhat toxic to chicks ; but, in those which were resistant, there was a considerable reduction in palpable tumours and an increase in survival time.

3. A-methopterin (4-amino-10-methylpteroylglutamic acid) given intraperitoneally to chicks, bearing either tumour strain RPL 12 or RPL 19, in doses of 50 or 100 μg./day was less toxic than aminopterin and exhibited some tumour-inhibiting action. The toxicity was completely counteracted by *citrovorum* factor administered intraperitoneally, but the tumour-inhibiting action was neutralized.

4. A-methopterin (10 mg./kg. of diet) was somewhat toxic, but in resistant chicks bearing RPL 12 there was a reduction in the number of palpable tumours and an increase in survival time. In chicks bearing RPL 19 this level was ineffective, but on raising it to 20 mg./kg. of diet a tumour-inhibiting effect was found.

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References

Bennette, J. G. (1952). *Brit. J. Cancer*, **6**, 377.

Bessis, M., and Freixa, P. (1950). *C.R. Soc. Biol., Paris*, **144**, 885.

Craigie, J. (1949). *Brit. J. Cancer*, **3**, 249.

Darcel, C. le Q. (1953a). *Brit. J. Pharmacol.*, **8**, 278.

—— (1953b). *J. comp. Path.*, **63**, 112.

Little, P. A., Sampath, A., Paganelli, V., Locke, E., and Subbarow, Y. (1948). *Trans. N.Y. Acad. Sci.*, **10**, 91.

Ringsted, J. (1952). *Acta path. microbiol. scand.*, **31**, 275.

—— (1953). Ibid., **33**, 44.

Sauberlich, H. E. (1953). *J. Nutr.*, **50**, 101.

—— and Schaefer, A. E. (1954). *Poult. Sci.*, **33** 107.

Stock, C. C., Biesele, J. J., Burchenal, J. H., Karnofsky, D. A., Moore, A. E., and Sugiura, K. (1950). *Ann. N.Y. Acad. Sci.*, **52**, 1360.

Woll, E. (1948). *Trans. N.Y. Acad. Sci.*, **10**, 83.