FURTHER INVESTIGATIONS ON THE MODE OF ACTION OF SUBSTANCES INHIBITING TUMOR GROWTH AND ON IMMUNIZATION AGAINST THESE SUBSTANCES.*

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The results which we obtained in our previous investigations on the mode of action of substances inhibiting tumor growth[1] were based partly on statistical methods; we therefore hesitated in our previous communications to pronounce our results as definite. The importance of these results, however, seemed to us to call for further experiments which should test the validity of our conclusions and extend the experimental basis on which they rested. In order to confirm these experiments we used a large number of mice, 604 of which lived to the end of the experiments and are included in our report.

THE SPECIFICITY OF THE IMMUNIZING ACTION OF INJECTIONS OF HIRUDIN AND COLLOIDAL COPPER.

In our previous paper[2] we concluded from the results of experiments carried out with the first method that the immunity against the influence of colloidal copper and hirudin in inhibiting tumor growth conferred through several injections of these substances, is, in the main, specific; that injections of colloidal copper immunize principally against the effects of colloidal copper and not, or only to a slight extent, against those of hirudin, and that hirudin immunizes principally against the effects of hirudin.

It was much to be desired that these conclusions should be further tested by means of the more exact second method. We made, there-

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[1] Fleisher, M. S., Vera, M., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 522.

[2] Fleisher, Vera, and Loeb, *loc. cit.*

fore, two additional experiments, the results of which are given in table I in horizontal columns 3 and 4.

TABLE I.

	No. of mice.	α	β	γ	δ	a	c	d	f	Horizontal column.
E–1 Controls	22	—	—	80%	—	—	4 81%	10 83%	8 75%	1
E–1 Copper controls: 9th–13th dy.	51	66%	55%	58%	60%	8 96%	14 55%	18 59%	11 20%	1
E–1 Immunized: 2d–6th dy. Copper: 9th–13th dy.	52	98%	87%	88%	91%	8 118%	12 84%	19 77%	13 74%	1
E–2 Controls	18	138%	144%	136%	139%	3 151%	2 125%	7 136%	6 127%	2
E–2 Copper controls: 9th–13th dy.	64	87%	81%	78%	83%	7 72%	9 76%	21 74%	27 92%	2
E–2 Copper series: transplanted 9th–13th dy.	70	138%	135%	134%	136%	10 121%	20 117%	24 147%	16 150%	2
F Controls	31	158%	158%	167%	161%	7 176%	13 134%	7 170%	4 188%	3
F Hirudin: 2d–6th dy. Hirudin: 9th–13th dy.	72	157%	149%	152%	153%	11 173%	19 148%	26 143%	16 145%	3
F Hirudin: 2d–6th dy. Copper: 9th–13th dy.	72	123%	117%	122%	120%	11 163%	24 108%	24 112%	13 105%	3
G Controls	28	141%	149%	150%	147%	—	6 153%	8 149%	14 147%	4
G Copper: 2d–6th dy. Hirudin: 9th–13th dy.	61	76%	75%	73%	74%	—	8 59%	23 98%	30 62%	4
G Copper: 2d–6th dy. Copper: 9th–13th dy.	63	150%	136%	147%	144%	—	6 175%	22 134%	35 131%	4

α Percentage of increase on comparing the sum of the original weight of all tumors (calculated) with the sum of the end weight (weighed).

β Multiply the average percentage of increase of each class (a, c, d, f) by the number of mice in that class, add the figures thus obtained, and divide the sum by the total number of mice used in that series.

γ Average percentage of increase of the various classes; add the percentage increases in each class and divide by the number of classes.

δ Average of the three preceding averages.

In the tables the series in each column which have the same mark, for instance A1 and B1, represent experiments done under approximately the same conditions and at the same time.

The figures above and to the left of the main figures indicate the number of mice used in each class.

In the experiment recorded in column 3, 175 mice lived until the end of the experiment; 31 served as controls; 144 mice received from the second to the sixth day a daily injection of hirudin, each animal thus receiving four preliminary (preparatory) injections. Of these 144 mice, one-half received from the ninth to the thirteenth day injections of hirudin, while the second half received colloidal copper. The same doses of these substances were used as in our previous experiments.[3] According to the weight of the mouse tumors on the ninth day, we divided them into the same four lots (a, c, d, f) as in the previous experiments. Such a division is necessary because the rapidity of growth of a tumor in a given period is, among other factors, as a rule, a function of its weight. In these experiments the difference in the various classes happens to be slight. The increase in weight in the different classes from the ninth to the thirteenth day is given in the vertical columns. In the vertical columns, α, β, γ, δ, the average of the increase in weight of the tumors of all the mice treated in the same manner is given, calculated according to the four different procedures which have been described in our previous paper.[4] We find that the mice which had been treated with injections of hirudin from the second to the sixth day were only slightly affected by the second set of injections of hirudin. The figures showing the percentage increase in weight from the ninth to the thirteenth day, during which period the animals received injections of hirudin, were only slightly less than those of the controls; while, on the other hand, the mice which received during this same period injections of colloidal copper were noticeably inhibited in their tumor growth. We may therefore conclude that while the preliminary injections of hirudin immunized the animals almost completely, or at least very markedly, against the effect of later injections of hirudin, they did not exert such an effect towards

[3] Fleisher, Vera, and Loeb, *loc. cit.*

[4] Fleisher, Vera, and Loeb, *loc. cit.*

later injections of colloidal copper, in so far as the immunizing effect of preliminary injections of hirudin is a specific one. This conclusion is confirmed by the result of the experiment in horizontal column 4. 124 animals received from the second to the sixth day after inoculation daily intravenous injections of colloidal copper; 63 of these mice received again colloidal copper from the ninth to the thirteenth day; while 61 mice received hirudin from the ninth to the thirteenth day. In the first lot, immunized against the action of colloidal copper, the colloidal copper exerted no, or only a very weak inhibiting action on the tumors, which grew therefore almost as well as in the controls; while in the second lot, which was again immunized against colloidal copper, but not against hirudin, injections of hirudin exerted a marked inhibiting action on tumor growth. This experiment at the same time proves that the hirudin used in the experiment recorded in horizontal column 3 would have been sufficiently strong to exert an inhibiting effect in the latter experiment, provided that the mice had not been immunized against the inhibiting action of hirudin. We may, therefore, on the basis of these, and of our previously recorded experiments, conclude that the immunizing action of preliminary injections of hirudin and colloidal copper is specific.

THE IMMUNIZATION OF TUMOR CELLS AGAINST THE EFFECT OF COLLOIDAL COPPER AND THE TRANSMISSION OF THIS IMMUNITY TO SUCCEEDING CELL GENERATIONS.

Further experiments were carried out in order to test and, if possible, to confirm our previous results, which indicated that the immunity produced against substances inhibiting tumor growth resides partly in the tumor cells and is transmitted to succeeeding generations of tumor cells. In horizontal column 2, table I, an additional experiment of this kind is recorded. 152 animals lived to the end of the experiment; 18 were controls, not injected animals; all the others received from the ninth to the thirteenth day injections of colloidal copper; 70 of these 134 mice had been inoculated with pieces from tumors grown in mice which had received injections of colloidal copper from the second to the sixth day and from the ninth to the thirteenth day. An immunity had therefore presumably been

established in the tumor cells against the effect of colloidal copper. That this is the case is shown in horizontal column 2. In the last lot of 70 mice, the tumors grew approximately as well as in the controls, despite the injections of colloidal copper which these 70 mice received, while in the 64 mice with non-immunized tumors, the tumor growth was inhibited to the usual extent through the injections of colloidal copper given from the ninth to the thirteenth day. In addition we carried out another experiment in which is compared the effect of colloidal copper on tumors in 52 animals which had been immunized against the effect of colloidal copper through preceding injections with this substance, given from the second to the sixth day, and in 51 animals which had not received a series of preliminary injections. We see that in the immunized animals the tumors grew about as well as in the non-injected controls, while in the non-immunized animals the tumor growth was inhibited through colloidal copper. These results again confirm our previous conclusions.

THE INEFFICACY OF INJECTIONS OF COLLOIDAL COPPER AND OF HIRUDIN ON VERY YOUNG TUMORS.

As we have observed previously, the weight of a tumor on the ninth day after transplantation usually enters as a factor into the set of conditions which determine the increase in weight of the tumors in the following four days. In order to rule out a possible influence of this factor on the results of our experiments, we had to compare on the ninth day the average weight of the various lots of tumors, arranged in the various classes. Tumors of smaller weight usually show in the following period from the ninth to the thirteenth day a greater increase in weight than tumors of larger weight. We must examine whether the differences in the weight of the various lots correspond and can be held responsible for the differences observed in weight increase from the ninth to the thirteenth day in immunized and non-immunized mice. Such a table should also give evidence of a possible inhibiting effect of intravenous injections of colloidal copper or of hirudin given from the second to the sixth day. If such an inhibiting effect should exist, the average weight of tumors on the ninth day in mice which received these preliminary

TABLE II.

Weight of Various Classes of Tumors at Nine Days.

Column 1. Normal uninjected controls.				Column 2. Mice injected with colloidal copper 9th–13th dy.				Column 3. Mice injected with colloidal copper 2d–6th and 9th–13th dys.				Column 4. Mice inoculated with tumor from mice injected with colloidal copper. Injected with colloidal copper 9th–13th dy.			
a	c	d	f	a	c	d	f	a	c	d	f	a	c	d	f
H				A–1								A–1			
				5		9	10						18	12	21
				—	286	176	58					—	247	173	51
D				A–2				A–2				A–2			
4	8	35	36		6	5	23		5	7	6	—			
367	240	158	92	—	283	198	68	—	270	162	87				
B–1				B–2				B–1							
1	6	6	6	17	30	8	4	12	26	14	8				
350	271	180	91	462	281	177	106	466	291	186	92				
B–2				B–2								B–2			
10	6	5	18	10	14	12	13					9	22	18	13
525	291	137	72	446	297	156	92					421	280	160	60
C–1				C–1				C–1							
3	12	3		14	15	14	15	4	16	19	16				
532	276	190	—	439	293	162	87	447	291	180	84				
C–2												C–2			
		15	35	8	20	15	23					6	22	22	18
—	—	143	68	476	284	156	73					411	292	159	101
E–1				E–1				E–1							
	4	10	8	8	14	18	11	8	12	19	13				
—	308	179	96	434	264	176	94	380	265	162	92				
E–2				E–2								E–2			
3	2	7	6	7	9	21	27					10	20	24	16
426	285	166	73	413	277	164	85					426	292	167	94
Controls:				F Hirudin-copper or		G Copper-copper		F Hirudin-hirudin or		G Copper-hirudin					
F Specimen 1				F				F							
7	13	7	4	11	24	24	13	11	19	26	16				
431	294	180	110	437	294	185	103	415	293	175	104				
G Specimen 2				G				G							
6	8	14			6	22	35		8	23	30				
—	267	153	94	—	263	149	95		255	147	94				

The letters and numbers of each group in table II correspond to the designations in table I of this paper and table I of our previous paper.[5] By comparing this table with the two other tables, it will therefore be possible to determine the increase in weight from the ninth to the thirteenth day of the tumors of the various classes of mice.

[5] Fleisher, Vera, and Loeb, *loc. cit.*

injections should be less. We notice that the animals which received injections of colloidal copper or of hirudin from the second to the sixth day had on the ninth day tumors of approximately the same weight as mice without such injections. In column 3, table II, is given the average weight of the tumors of the various lots of mice in classes, a, c, d, f, according to the weight limits fixed in our previous paper. We see that there is no noticeable difference in the average weight of the different classes of mice, if we compare columns 3 and 2. In the latter is the weight of the tumors of mice which had not received the preliminary injections. In the last two horizontal columns (F and G) we can compare the weight on the ninth day of tumors of non-injected mice (vertical column 1) with those having received hirudin (F vertical column, Nos. 2 and 3) and colloidal copper (G vertical column, Nos. 2 and 3) from the second to the sixth day. We see that on the whole there is no marked difference in the weight of the tumors of the different lots of mice.

In vertical column 4 we find a few classes of tumors with somewhat smaller weight; namely, c and f in horizontal column A^1, and in group F in horizontal column B^2. All others are approximately normal. If we analyze the figures indicating the increase in weight of the tumors from the ninth to the thirteenth day of mice inoculated with immunized tumor material, consulting for this purpose the increase of weight of these tumors given in table I of our previous paper[6] and in table I of this paper, we see that our conclusion as to the lack of inhibition in the growth of tumors of these lots of mice is not dependent on these few classes, but is noticeable in other classes as well. We may therefore conclude that the immunization which we observed in mice having received a preliminary set of injections, either from the second to the sixth day after inoculation, or in the previous generation of mice, is real and is not due merely to the difference in weight of the tumors on the ninth day. We may furthermore conclude that injections of colloidal copper or hirudin at an early stage (from the second to the sixth day) after transplantation do not inhibit tumor growth to a noticeable extent.

[6] Fleisher, Vera, and Loeb, *loc. cit.*

THE EFFECT OF A COMBINATION OF HIRUDIN AND COLLOIDAL COPPER ON TUMOR GROWTH.

In our previous paper we reported that combinations of colloidal copper and hirudin and of colloidal copper and nucleoproteid were much more effective than either of these substances alone, and that these combinations not only caused an inhibition of the tumor growth but also retrogression of a considerable number of tumors; we also found that the combination of colloidal copper and hirudin was more toxic than either substance given alone. In order to confirm this result we carried out an additional experiment of which table III gives the results.

TABLE III.

Substance injected.	Dose.	Number of tumors and mortality.	Result.
Combination of hirudin and copper	0.0125 to 1.25 mg. 0.25 c.c.	27 tumors; mortality 29% (8 died)	4 grew well, 1a, 1c, 2d; 15%. 8 were retarded, 2a, 3c, 3d; 30%. 15 retrogressed, 3a, 6c, 2d, 4f; 55%.
Controls	.....	30 tumors	1 did not grow, 1c; 3%. 29 grew well, 7a, 12c, 7d, 3f; 97%.
Hirudin	0.125 to 1.25 mg.	60 tumors; mortality 15% (9 died)	18 grew well; 30%. 32 were retarded; 53%. 2 retrogressed; 3%. 10 doubtful; 17%.

In the results we state the number of tumors in each class (a, c, d, f) of tumors.

Mice were injected with a mixture of 0.25 of a cubic centimeter of colloidal copper and 0.0125 of a milligram to 1.25 milligrams of hirudin, the smaller dose of hirudin being injected first, and the dose being gradually increased in the course of the following injections; while in the mice injected with hirudin alone 30 per cent. of the tumors grew normally, 17 per cent. were doubtful, 53 per cent. were retarded, and only 3 per cent. retrogressed (the latter being in this case included among the non-growing tumors); only 15 per cent. of tumors grew normally among the mice treated with the combination of hirudin and colloidal copper; 30 per cent. were retarded, and 55 per cent. retrogressed. Among this latter lot of mice there had been

a mortality of 29 per cent. (only the mice living to the end of the experiment being included in the list); among the mice injected with hirudin only, the mortality was smaller; *viz.*, 15 per cent. We see, therefore, that the combination of hirudin and colloidal copper is much more effective than either substance given alone.

These additional investigations confirm, therefore, our previous experiments, and we may now with still greater certainty draw the general conclusions which we mentioned in our previous paper on immunization against the action of substances inhibiting tumor growth.

SUMMARY.

Our later investigations confirm our previous results.

1. It is possible to increase markedly the effect of substances inhibiting tumor growth by using certain combinations of these substances which, when given alone, have some effect on tumor growth.

2. Immunity acquired against the effect of these substances depends partly upon an active immunization of the tumor cells themselves against the action of these substances, and this immunity is transmitted to the following generations of tumor cells.

3. The immunity against the substances inhibiting tumor growth is, as far as we have investigated the problem, specific.

4. Our later experiments provide a more secure basis for the additional and more general conclusions which we mentioned tentatively in our previous paper.