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UNITED STATES ATOMIC ENERGY COMMISSION

"ISOLATION OF A FACTOR PREVENTING X-RAY INDUCED LEUKEMIA"

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SUMMARY:

This document contains the results of fractionation and leukemia-prevention studies begun and partially completed on the proteins of Cohn Fractions III and IV obtained from sheep serum, and on studies with other proteins of ovine origin. Each of these proteins has been tested for its ability to prevent lymphosarcoma (leukemia) in x-irradiated C57Bl/6 mice. The same caveat offered in previous years must be repeated. Each experiment takes well over a year, and this report covers experiments begun before the period dates and completed during the current period, as well as experiments the results of which will be manifest only in the next year or so.

Work on development of a shorter assay for RLP (radiation-leukemia protection factor) was continued. Spleen and serum fractions were assayed for α_2 -macroglobulin (as the trypsin-esterase complex resistant to inhibition by soybean trypsin inhibitor). A good correlation between RLP activity and α_2 M content was found for spleen but not for serum. Changes in enzyme levels after x-irradiation and treatment with RLP were studied. DNA and RNA polymerase were added to the list of enzymes investigated. RNA

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polymerase behaves like the enzymes previously studied. By contrast, DNA polymerase decreases after irradiation and increases again when RLP is injected. Changes in the total peripheral leukocyte count and in thymus weight follow x-irradiation and are prevented in part by RLP. Finally, studies were begun on changes in the α_2 -M of serum after irradiation of the mouse and treatment with RLP. Here too, there are significant differences between irradiated and irradiated plus RLP treated mice. Several protein fractions are being compared in both the leukemia protection test and one or more of the shorter assays.

BIBLIOGRAPHY

1. COO-1391-10. A greatly modified form of this report has been accepted for publication in Cancer Research. The paper, by D. B. Clewell; J. D. Hubbard, P.-L. Yu, L. C. Yip and M. E. Hodes, is entitled FRACTIONATION OF SPLEEN AND SERUM IN ATTEMPTS TO ISOLATE THE RADIATION LEUKEMIA PROTECTION FACTOR.
2. COO-1391-11. CHANGES IN THE ENZYME LEVELS OF MOUSE ORGANS AFTER LEUKEMOGENIC DOSES OF X-RAY AND TREATMENT WITH RLP (RADIATION LEUKEMIA PROTECTION) FACTOR, by L. C. Yip, N. Blair, P.L. Yu and M. E. Hodes has appeared in the Israel J. Med. Sciences 4:1188 (1968).
3. COO-1391-14. CHROMOSOMAL STUDIES OF CELL POPULATIONS OF IRRADIATED MICE TREATED WITH RADIATION LEUKEMIA PROTECTION FACTOR, by C. G. Palmer, P.-L. Yu, and M. E. Hodes (abstract)

J. Cell Biology 39:174s (1968).

4. COO-1391-15. EXPERIMENTS ON THE MODE OF ACTION OF THE RADIATION LEUKEMIA PROTECTIVE ("RLP") ACTIVITY OF SERUM, by L. Yip, P.-L. Yu and M. E. Hodes (abstract). Proc. Am. Assoc. Cancer Res. (in press) 1969.
5. COO-1391-16. COMPARISON OF SOME PROPERTIES OF THE RADIATION-LEUKEMIA PROTECTION ("RLP") FACTORS OF SHEEP SPLEEN AND SERUM, by L. C. Yip and M. E. Hodes (abstract). Fed. Proc. (in press) 1969.

A. TESTING FOR RADIATION-LEUKEMIA PROTECTION IN C57B1/6 MICE.

This continues to be the major effort under the contract. The source of supply of mice, handling, x-irradiation, pathological and statistical examinations remain as previously reported, with only one change in personnel in the animal room. Most of the difficulties reported before with *Pseudomonas* infection have been overcome, and survivals are now generally good. The results of the leukemia tests are summarized below and in Tables 1-5. Final autopsy reports are not yet available, and only *P* values based on χ^2 are given.

(1) Sheep hemoglobin and its derivatives.

Those experiments were begun because some of the active fractions obtained in the early experiments seemed to contain hemoglobin. The results are summarized in Table 1.

Sheep hemoglobin (commercial) appears to contain RLP, whereas the derivatives are either negative or inconsistently positive. Several additional experiments are still too early to evaluate (Table 5)

(2) Commercial fractions of sheep serum.

The experiments summarized here and in Table 3 of COO-1391-13 indicate that some of the Cohn fractions possess leukemia-protection activity. The chromatographic studies reported below were started in consequence of these preliminary results. See Table 2 for summary

(3) DEAE cellulose chromatography of Cohn fraction IV.

The only promising fraction in the entire experiment was No. 6 (Table 3). Unfortunately, several groups of mice, including the repeat on 6, were terminated prematurely. This will be scheduled for retesting.

The elution pattern from DEAE-cellulose is shown in Fig. 1 of COO-1391-13.

(4) DEAE-cellulose chromatography of Cohn fraction III.

Column fraction 5 (Table 4) has shown activity twice. The remainder of the fractions, with the possible exception of 3, appear negative.

The elution diagram is shown in Fig. 1, the polyacrylamide gel electrophoresis patterns in Fig. 2, and the immunoelectrophoretic patterns in Fig. 3.

(5) Materials entered in lymphosarcoma tests less than 8 months prior to report. These are listed in Table 5. The results should be available next year.

The possibility that maximal RLP activity may require a combination of fractions is discussed in the article to be published in Cancer Research. Further work along this line awaits the results on the fractions now in test.

B. ATTEMPTS TO DEVELOP A SHORTER ASSAY FOR RLP

(1) Berenblum and his associates recently reported that (human) α_2 -macroglobulin possesses RLP activity (Nature 217:857, 1968). α_2 -macroglobulin (α_2 M) is able to bind trypsin to form a complex that retains about 75% of the esterase activity of unbound trypsin. This complex is not subject to inhibition by soybean trypsin inhibitor. We have used a modification of the technique of Ganrot (Clin. Chim. Acta 14:493, 1966) to determine the α_2 M (as trypsin esterase complex) of serum fractions. Benzoyl-DL-arginine-p-nitroanilide was the substrate. Hydrolysis was followed continuously in the Gilford spectrophotometer at 410 m μ at 25° C. Results are reported as change in A_{410} (ΔA_{410}) per minute per ml added fraction. A correlation between α_2 M and activity in the leukemia prevention test is shown in Table 6. The data in the table show that whereas the α_2 M activity of spleen extracts and the fractions prepared from them demonstrate a good correlation with RLP activity, this correlation does not hold for sheep serum. Therefore, the test for α_2 M in fractions is not satisfactory as a short assay for RLP activity. The fractionation of serum and spleen on the same Sephadex column is shown in Figs 4 and 5. The α_2 M elutes in the macroglobulin region.

(2) Enzyme changes after irradiation alone or irradiation and treatment with RLP.

The early experiments were reported in COO-1391-4, 11 and 12. The work summarized here has been done in the last year.

(a) DNase II. The changes in serum levels of DNase II were measured in Swiss mice after various doses of radiation and treatment with

sheep serum or saline. The results are summarized in Table 7. There is generally a rise on the 2nd or 3rd day after x-ray, and this may be very slightly less after sheep serum injection. The experiments should be repeated using partially purified DNase II.

(b) DNA polymerase. Regeneration of thymus tissue after x-irradiation is promoted by RLP (Cividalli and Kruszynski, Radiation Res. 30:148, 1967). The incorporation of labelled thymidine into bone marrow of irradiated mice is enhanced by RLP treatment (Berenblum *et al.*, Nature 217:857, 1968). It appeared likely that DNA polymerase activity would be higher in irradiated, RLP treated animals than in irradiated controls. An experiment was designed to test this hypothesis. In part one of the experiment, three groups of Swiss mice were exposed to 400 R once (groups A and B) or maintained as age controls (group C). Group B also received RLP (sheep serum). The DNA polymerase activity of thymus and bone marrow was assayed on days 4, 5, 6 and 7 after irradiation.

For part 2 of the experiment only the x-ray dose was changed (from 400) to 300 R. For part 3, C57Bl/6 mice were exposed to 300 R. The results are shown in Fig. 6, 7 and 8. In contrast to the findings with the other enzymes studied (LDH, DNase II, alkaline phosphatase), DNA polymerase levels fell after irradiation. RLP appears to hasten the recovery of the polymerase values to normal. The differences among groups for part 3 (thymus) was found to be highly significant in an analysis of variance.

(c) RNA polymerase. Both Swiss and C57B1/6 mice were used in these experiments. Observations were made on the RNA polymerase activity of thymus and bone marrow. The results of these experiments are summarized in Figs. 9 and 10. An analysis of variance on the results obtained from the C57B1/6 thymus experiment reveal a highly significant difference among groups. In contrast to the findings with DNA polymerase, the results indicate a rise in RNA polymerase activity after x-ray and a decrease after RLP treatment.

(3) Changes in thymus weight.

In the aforementioned experiments of Cividalli and Knyszynski, increases in thymus weight appeared sooner in mice x-irradiated and treated with RLP. The C57B1/6 mice were subjected to the usual leukenogenic regimen (180 R per week four times) and the full course of RLP. Recovery was seen some weeks after treatment. Our experiments were performed on C57B1/6 and Swiss mice. (These were used for the polymerase experiments reported above). A single exposure to 300 R was followed by a single injection of RLP (sheep serum). The overall differences among groups of Swiss mice was not significant in an analysis of variance, but the results for C57B1/6 reveal a consistent and significant difference from day 3 on (Fig. 11). This result is very promising.

(4) Changes in white blood count.

Dr. D. Uphoff suggested to us that changes in white blood count (WBC) might be a useful index of RLP activity (see Lorenz, Congdon and Uphoff, J. Natl. Cancer Inst. 14:291, 1953). Preliminary results indicate

a highly significant difference between groups beginning about day 16 after irradiation (Fig. 12) and injection of serum. This work is being pursued.

(5) Changes in serum α_2 -macroglobulin (α_2M).

The role of α_2 -macroglobulin in serum is not clear. As mentioned above, Berenblum suggests that RLP is α_2M , whereas our results are not entirely consistent with this conclusion. These observations aroused our interest in the role of α_2M in radiation leukemia and have led to intensive studies of the α_2M of protective fractions from sheep spleen and serum (reported above) and of variations in α_2M in mouse serum after irradiation (see below)

(a) Comparison of human and mouse α_2M by immunoelectrophoresis. Fig. 13 shows clearly that mouse serum contains a protein similar but not identical to human α_2M . This confirms the observation of Hanna *et al.*, (Science 157:1458, 1967) that mouse serum contains α_2M , and casts doubt on the claim (Heilm, Nature 217:1057, 1968) that rats - and other rodents - do not normally have α_2M .

(b) Changes in mouse serum trypsin-binding activity after x-irradiation and RLP treatment. Both Swiss and C57B1/6 mice were examined. Mice were bled on days 2, 4, 7, 9, 11 and 14 (Swiss) or 1, 3, 5, 7, 9, 11 and 14 (C57B1/6) after irradiation. The results are shown in Fig. 14 and 15. The trypsin binding activity for RLP treated animals was significantly higher than for x-irradiated alone or albumin injected mice. The significance of this finding is under investigation.

C. STUDENTS TRAINED

Dr. Lily C. Yip finished her postdoctoral training and was promoted to Visiting Instructor in Biochemistry.

Darlene Sayers, a medical student, worked on the enzyme test during the summer of 1968.

Theresa Nong, a graduate student, studied changes in serum LDH during the Fall Semester, 1968.

Fig. 1. Fractionation of sheep serum commercial Cohn fraction III on DEAE-cellulose. Column size, 7 x 66 cm; flow rate 15 ml/min; fraction size, 23 ml. Stepwise elution with 0.01 M phosphate buffer containing NaCl as indicated. Starting pH 8.0. The method is that of Oh *et al.*, Anal. Biochem. 16:220 (1966).

SS C III 1% 2 3 4 5 6

7 8 9 10 11 12 13 14

Fig. 2. Electrophoresis in polyacrylamide gel of the pools obtained after chromatography of Cohn III on DEAE-cellulose (See Fig. 1). SS, sheep serum; C III, Cohn III, 1%: fl-14, pools 1-14.

Fig. 3. Immunoelectrophoresis of the pools obtained after chromatography of Cohn III on DEAE-cellulose (See Fig. 1). 1% agar, pH 8.6, 3 hours, 14 mamp per plate.

Fig. 4. Fractionation on Sephadex G-200 of precipitate obtained after 0-40% saturation of sheep serum (A) or spleen extract (B) with ammonium sulfate. Column size, 1 x 78 cm; flow rate, 7.2 ml/min; sample size, 5 ml; fraction size, 2.2 ml; void volume, 88 ml.

Fig. 5. Fractionation on Sephadex G-200 of precipitate obtained after bringing to 60% saturation with ammonium sulfate of sheep serum (A) or spleen extract (B) supernatant from 0-40% saturation step. For details on column and elution, see Fig. 4.

BONE MARROW DNA POLYMERASE AFTER 300R

FIG 6

FIG 7

THYMUS DNA POLYMERASE AFTER 300 R

FIG. 8

THYMUS RNA POLYMERASE AFTER 300 R

FIG. 9

THYMUS RNA POLYMERASE AFTER 300R

FIG. 10

THYMUS WEIGHT AFTER 300 R

FIG. 11

WHITE BLOOD CELL COUNT AFTER 300 R

FIG. 12

- + goat antihuman α_2M
- + human plasma
- + goat antihuman α_2M
- + human plasma + mouse serum
- + rabbit antimouse serum
- + mouse serum
- + rabbit antimouse serum

Fig. 13 Relationship between α_2M of human and mouse sera as determined by immunodiffusion in agar.

γ_2 M-TPE ACTIVITY OF SERUM AFTER 300R

α_2 M-TPE ACTIVITY OF SERUM AFTER 300R

Table 1

Preliminary results obtained in the leukemia prevention test
with sheep hemoglobin or its derivatives

Expt. No.	Material injected	Lymphosarcomas		P	Duration (days)
		Survivors at first lymphosarcoma			
74	Hgb, 0.05% PBS	4/22 31/40		.001	365
86	Hgb, 0.05% PBS	16/37 14/42		n.s.	"
102	Hgb, 0.05% PBS	9/36 17/31		.05	"
103	Hgb, 0.025% PBS	16/46 25/43		n.s. ¹	"
104	Denat. hgb, 0.025% PBS	11/44 16/45		n.s.	"
105	Hemin, 0.025% PBS	5/46 20/39		.001	"
106	Globin, 0.025% PBS	11/42 14/44		n.s.	348
108	Globin, 0.05% PBS	6/36 13/45		n.s.	348

¹at borderline of significance

Table 2
Preliminary results obtained in the leukemia prevention
test with commercial Cohn fractions¹

Expt. No.	Material injected	Lymphosarcoma	P	Duration (days)
		Survivors at first lymphosarcoma		
84	Fr. IV, 0.5% PBS	8/33	.05	365
85	Fr. IV-4, 0.5% PBS	12/34 12/44	n.s.	365
107	Fr. I, 0.5% PBS	8/49 25/42	.001	349
109	α_2 -globulin, 1% PBS	3/44 29/44	.001	332
110	α_2 -globulin, 1% PBS	3/49 8/50*	n.s.	330

¹ See also Table 3 of COO-1391-13

* This is an unusually low control incidence

Table 3

Preliminary results obtained in the leukemia prevention test with Cohn fraction IV chromatographed on DEAE-cellulose column.

Expt. No.	Material injected	Lymphosarcomas	P	Duration (days)
		Survivors at first lymphosarcoma		
88	Fr. 1 PBS	*		--
89	Fr. 2 PBS	*		--
90	Fr. 3 PBS	15/42 14/44	n.s.	365
91	Fr. 4 PBS	3/30 8/41	n.s.	"
92	Fr. 5 PBS	9/32 12/32	n.s.	"
93	Fr. 6 PBS	7/31 18/33	.05	"
94	Fr. 1 PBS	*		"
95	Fr. 2 PBS	11/43 22/47	n.s.	"
96	Fr. 3 PBS	13/46 17/49	n.s.	"
97	Fr. 4 PBS	*		"
98	Fr. 5 PBS	*		"
99	Fr. 6 PBS	*		"
100	Fr. 7 PBS	8/36 9/37		"
101	Fr. 8 PBS	16/40 22/37		"

* Terminated early owing to excessive mortality

Table 4

Preliminary results obtained in the leukemia prevention test
with Cohn fraction III chromatographed on DEAE-cellulose columns

Expt. No.	Material injected	Lymphosarcomas		P	Duration (days)
		Survivors at first lymphosarcoma			
111	Fr. 1 PBS	*			--
112	Fr. 2 PBS	*			--
113	Fr. 3 PBS	9/44 20/41		0.05	305
114	Fr. 4 PBS	15/42 16/45		n.s.	304 304
115	Fr. 5 PBS	3/26 13/35		0.05	289 289
116	Fr. 6 PBS	18/45 15/37		n.s.	288 288
117	Fr. 7 PBS	11/32 18/37		n.s.	276 276
118	Fr. 8 PBS	21/44 20/39		n.s.	275 275
122	Fr. 1 PBS	23/45 22/46		n.s.	247 247
123	Fr. 2 PBS	19/50 22/49		n.s.	231 231
124	Fr. 3 PBS	15/45 19/41		n.s.	230 230
125	Fr. 4 PBS	16/44 20/43		n.s.	220 220

Table 4 continued

126	Fr. 5 PBS	8/47 20/45	0.05	219 219
127	Fr. 6 PBS	*	n.s.	--
128	Fr. 7 PBS	4/49 1/47	n.s.	203 203
129	Fr. 8 PBS	7/46 10/42	n.s.	191 191
130	Fr. 9 PBS	1/47 6/40	n.s.	190 190
131	Fr. 10 PBS	*	n.s.	--
132	Fr. 11 PBS	2/47 3/42	n.s. n.s.	176 176
133	Fr. 12 PBS	*	n.s.	--
134	Fr. 13 PBS	*	n.s.	--
135	Fr. 14 PBS	1/34 1/49	n.s.	156 156

* Terminated early owing to excessive mortality

Table 5

Materials entered in leukemia prevention tests
of less than 8 months duration

Experiment No.	Material injected ¹
120	Mouse tissue extract
121	" " "
136	Sheep globulin, 0.1%
137	Hemin, 0.1%
138	Fibrinogen, 0.5%
139	Hemoglobin, 0.5%
140-153	Cohn III, DEAE fr. 1-14
154-167	Cohn IV-4, DEAE fr. 1-14

¹ 50 mice entered in each test

Table 6

The trypsin binding activity of serum and spleen

A. Sephadex fractions

Spleen			Serum		
Material	Leukemia test	$\Delta A_{410}/\text{min/ml}$		Leukemia test	$\Delta A_{410}/\text{min/ml}$
A. 40% homogenate	+	1.6	a. whole serum	+	3.75
B. 40% amm. sulf. ppt. of A	+	10.0	b. 40% amm. sulf. ppt. of a	+	3.50
C. 40-60% amm. sulf. ppt. of A	+	10.0	c. 40-60% amm. sulf. ppt. of a	+	23.0
D. Sephadex fractions of B			d. Sephadex fractions of b		
Fr. 1	+	+	Fr. 1	-	+
Fr. 2	-	-	Fr. 2	-	-
Fr. 3	-	-	Fr. 3	-	-
Fr. 4	-	-	Fr. 4	+	-
Fr. 5	-	-	Fr. 5	-	-
E. Sephadex fractions of C			e. Sephadex fractions of C		
Fr. 1	+	+	Fr. 1	-	+
Fr. 2	-	-	Fr. 2	+	-
Fr. 3	-	-	Fr. 3	+	-
Fr. 4	-	-	Fr. 4	-	-
Fr. 5	-	-	Fr. 5	+	-

B. Whole serum and commercial Cohn fractions

Material	$\Delta A_{410}/\text{min/ml}$	% Whole serum activity
Mouse serum	3.22	--
Human plasma	2.52	100
Sheep serum (fresh)	3.40	--
Sheep serum (stored)	2.40	100
Sheep Cohn fr. I (1% solution)	0.022	0.9
II "	0.040	1.7
III-0 "	0.038	1.6
III "	0.150	6.3
IV-1 "	0.074	3.1
IV-4 "	0.012	0.5
IV "	0.092	3.5
V "	0.0	0
Human α_2 -globulin	0.026	1.03

Table 7

DNase II levels in mouse serum after x-irradiation

Treatment	Mouse no.	Days after x-irradiation		
		1	2	3
$\Delta A_{260}/10 \text{ min}$				
A. 800r + PBS	1	0.065	.470	.045
	2	0.060	.445	.073
	3	0.122	.334	--
B. 800r + SS	1	0.105	.307	.015
	2	0.129	.351	.022
	3	0.054	.415	.051
C. 300r + PBS	1	0.094	.013	.051
	2	0.117	.423	.057
	3	0.100	.066	--
D. 300r + SS	1	0.070	.247	.067
	2	0.103	.043	.025
	3	0.038	.044	.028
E. 180r + PBS	1	0.041	.175	.210
	2	0.152	.366	.063
	3	0.184	.210	.060
F. 180r + SS	1	0.136	.057	.297
	2	0.222	.170	.054
	3	0.054	.297	--
G. No treat; age control	1	--	.036	.015
	2	--	--	.023
	3	0.006	.037	.056