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PROGRESS REPORT

UNITED STATES ATOMIC ENERGY COMMISSION

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

MASTER

Period Covered: 1 February 1969 through 31 December 1969Principal Investigator: M. E. Hodes, M.D., Ph.D.
Professor of Medicine and BiochemistrySUMMARY:

This document presents the results of fractionation and leukemia-prevention studies begun and fully or partially completed on the proteins of Cohn fractions III, IV, and IV-4 and of other proteins of various origins. Each of these proteins has been tested for its ability to prevent lymphosarcoma (leukemia) in x-irradiated C57Bl/6 mice. The summary includes experiments begun several years ago and now completed. It also includes work in progress, the final results of which will not be known for at least another year. It is still not possible to pinpoint the RLP (radiation leukemia prevention) activity, although some fractions have been active in preventing leukemia.

The results of attempts to develop a shorter assay for RLP are presented. In general, this approach has centered on the reversal of certain biochemical and physiological effects of a single dose of x-irradiation. The concentration of enzymes such as alkaline phosphatase, lactate dehydrogenase, DNase II and RNA polymerase rise after x-irradiation. Serum injections reverse the rise. DNA polymerase and alpha-2-macroglobulin (trypsin esterase binding activity) fall. Serum injections reverse this effect also. WBC and thymus weight fall after x-ray and rise again after serum injection,

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but the effect is delayed and not as striking as the enzyme effects. Attempts to correlate these findings with leukemia prevention, and to exploit them as an assay for RLP activity are continuing.

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A. TESTING FOR RADIATION-LEUKEMIA PROTECTION IN C57BL/6 MICE.

Over the years, this has continued to be the major effort under the contract. It consumes most of the time and the funds. The source of supply of mice, handling, x-irradiation, pathological and statistical examination remain much the same as previously reported. Because this is a triennial review, a brief recapitulation is presented here.

C57BL/6 mice are obtained from Laboratory Supply, a local breeder. They are inbred, but may be as much as 2 generations removed from the nucleus. They are obtained at 3 weeks of age, held in isolation by us for a week, then entered on study. (Previously mice were 6-7 weeks at start of experiment, but low incidences of leukemia in some controls, x-irradiated groups led us to favor younger animals). Mice are housed in plastic cages with steel tops and fed only pellets and chlorinated water, *ad lib*. Cages are changed 1-2 times per week. X-irradiation is from a 250 kv therapeutic

machine, through 0.5 mm Cu and 1 mm Al, target distance 70 cm, 43.1 r per minute. The mice receive 4 whole body doses of 188 r each (measured in air) at 7-day intervals. Injection of PBS (phosphate-buffered saline) or test substance, generally in 0.25 ml volume, is performed intraperitoneally within an hour of radiation.

Cages are inspected 1-2 times daily for dead or sick mice, and all animals are palpated once a week. Dead mice are autopsied and tumor, thymus, nodes, spleen, lungs, heart, liver and kidneys saved for histopathological examination. This work is done under the supervision of J. Donald Hubbard, M.D., pathologist.

All results are analyzed statistically by Dr. Pao-lo Yu, statistician. Generally the correction of Kimball (Bull. Internat. Statist. Inst. 36:193, 1958) for intercurrent deaths is applied. In most experiments 50 controls and 50 test animals of each sex are irradiated. (We have never been able to show interaction with sex, and the results are pooled for analysis).

The time from injection to autopsy is 365 days, as about 95% of the leukemias appear within this period. To this should be added at least a month on either end for preparation of material, autopsy and collation of data. For this reason, we have presented the data in these Progress Reports for all experiments, completed or not, stating tentative P values when final autopsy results were not available or when experiments were still in progress.

We will not present all the data gathered over 6 years, but summarize that which has appeared in Hodes *et al.*, Cancer Research 26:1780 (1966) and Clewell *et al.*, Cancer Research 29:1333 (1969), then give the results of experiments not included (that is, begun since accumulation of data for

those papers was stopped).

Sheep spleen, sheep serum and bovine serum contain a factor or factors (RLP) that decrease the incidence of leukemia in C57Bl/6 and BALB/C mice exposed to 4 doses of 188 r each of x-ray and injected after exposure. At the time this was demonstrated, no consistent protection against death could be demonstrated in C57Bl/6 or Swiss mice exposed to a single dose of 600-800 r. The RLP factor was absent from certain fractions of spleen and serum, such as albumin, and this fact prompted a search through the remaining fractions. Although RLP activity could be found in some fractions rather consistently, its exact identification has not yet been achieved.

There seem to be differences in the distribution of RLP in spleen and serum. In the former activity was found in the early peaks eluted from Sephadex ("19S" fraction), whereas the RLP activity in serum traveled with the lighter material. It was suggested that 19S, or alpha-2-macroglobulin (α_2M) might act as a carrier for RLP. In addition, examination of the data revealed that although statistically significant differences from control values could be found only with certain fractions, several neighboring fractions did exhibit evidence of protection. The scatter plus the discrepancy in distribution between sheep and serum suggested the alternative explanation of a multicomponent system. For obvious technical reasons, testing of the latter possibility has not been undertaken.

It must be emphasized that the extended test period, and the impossibility of predicting results before 7 or 8 months have passed, have forced us to test substances which seemed likely candidates before the final results of preliminary tests were available. Occasional positive results obtained with some of the commercial preparations we now tend to attribute

to contamination with RLP or random fluctuation. Whereas any significant difference has been cause for starting (duplicate) repeat experiments, results are not considered positive unless duplicates agree. The data presented below was obtained from experiments started after the preliminary results with certain commercial sheep serum preparations suggested RLP activity. Final results of these experiments are summarized in Clewell *et al.*, Cancer Research 29:1333 (1969). Final results of recent experiments with commercial sheep serum preparations are summarized in Table 1 of this report.

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

These results, obtained after chromatography on DEAE-cellulose of commercial Cohn fraction III from sheep serum, are summarized in Table 2. The experiments are marred by the early mortality in several groups and the occasional low incidence of leukemia in the control, irradiated group. There are rarely any apparent reasons for these interfering factors, and the only solution is repeated experiments. The differences in fractionation of these experiments is under investigation.

(2) DEAE-cellulose chromatography of Cohn fraction IV.

Results of the first two experiments were incomplete because of the early termination of some of the groups. Results are summarized in Table 3. Repeats are scheduled.

(3) DEAE-cellulose chromatography of Cohn fraction IV-4.

Cohn IV-4 has been fractionated on DEAE-cellulose by the same method used for Cohn III and Cohn IV. The elution pattern is illustrated in Fig. 1. Preliminary results of an experiment now nearing completion utilizing DEAE fractions of Cohn IV-4 are

summarized in Table 4. It appears in this single fractionation that RLP is concentrated in Fr. 13. Two repeats of this experiment are now in progress.

(4) Sheep hemoglobin and its derivatives.

These experiments were begun because some of the active fractions obtained in the early experiments contained hemoglobin. The results are summarized in Table 5.

(5) Materials entered in tests of less than 8 month's duration and miscellaneous experiments.

These are summarized in Table 6.

Conclusions: The possibility of isolating RLP from a subfraction of serum remains real. There are no serious obstacles other than time and money. RLP is present in several of the Cohn fractions, and particularly in subfraction 12 of Cohn III and subfraction 13 of Cohn IV-4. Our efforts will be centered around these subfractions.

B. BIOCHEMICAL CORRELATES OF RLP ACTIVITY

These studies, sandwiched in among the preparations and testing of the materials reported above, were motivated partially by the necessity of developing a shorter assay for RLP and partially by a desire to learn the mode of action of the factor.

(1) Enzyme levels.

The earlier experiments were based on the observation that the levels in serum and organs of a number of enzymes change after x-irradiation. We studied the effect of x-irradiation and injection

of serum or RLP-containing derivatives on the level of DNase II, alkaline phosphatase and lactate dehydrogenase in serum, liver, thymus and spleen. These experiments were published (COO-1391-11). They showed that injection of serum after x-irradiation could reverse the increase in serum and organ levels of these enzymes. Moreover, the experiments suggest that measurement of liver alkaline phosphatase or lactate dehydrogenase at 7 days after exposure to a single dose of x-ray and injection with test substance might serve as a short assay for RLP. Of course, the proof of this hypothesis rests in testing RLP against an inert substance (serum albumin, for example) and we are not yet in a position to make a definitive statement linking the enzyme-depressing and RLP activities of serum.

The enzyme studies were extended to include DNA and RNA polymerase. At the same time, it was decided to extend the study of serum effects to thymus weight and total peripheral white cell count. The reasons for these decisions were two-fold, and stemmed from a desire to extend our enzyme studies and to investigate the basis for the increased regeneration of thymus tissue after x-irradiation and injection of sheep spleen extract observed by Cividalli and Knyszynski (Radiation Research 30:148, 1967) and the enhanced incorporation of labeled thymidine into thymus after radiation and RLP-treatment observed by Berenblum *et al.* (Nature 217:857, 1968). The results of these experiments were published in part in COO-1391-17. It was found that while RNA polymerase behaved like the other 3 enzymes we had studied, rising after x-ray and falling after treatment with serum but not with saline or albumin, DNA polymerase showed an inverse

behavior. It fell after x-ray and saline injection and rose in mice x-irradiated and injected with serum.

The changes in thymus weight were also interesting. During the 14-day period investigated, there was a definite increase in the thymus weight of treated animals, but the difference from the controls did not reach the level of statistical significance. Similarly, there was a difference in peripheral wbc between the serum treated and other groups, but it reached significance only at the end of the test period.

These experiments have been extended during the past year. Obviously, the easiest parameters to measure are wbc and organ weight. The effect of several subfractions of Cohn III (see part A, above), serum albumin and saline were tested. All animals, except an age control group, received a single whole-body dose of 300 r x-irradiation. Spleen and thymus weights and wbc were measured from the 6th to the 21st day post irradiation. The results are briefly summarized below:

(a) Acute Expt. 17

Mice: Swiss, 4-5 weeks old, female

Test substances: PBS, albumin, Cohn III-subfractions 5 and 12;
(age controls). Four injections.

Results: No significant differences in spleen or thymus weight
or in wbc. Animals examined between days 6 and 21.

(b) Acute Expt. 18

Mice: C57B1/6, 4-6 weeks old, female

Test substances: PBS, Cohn III, Cohn III-subfractions 5 and 11;
(age controls). Four injections.

Results: No significant differences in wbc, thymus, spleen,
or body weight.

In this experiment an additional control was added: One group
received no x-ray but was injected with Cohn III. It did not
differ from the age control. Animals examined days 8-21.

(c) Acute Expt. 19

Mice: Swiss, 4-5 weeks old, female

Test substances: PBS, Cohn III-subfractions 5 and 13: (age
controls). Four injections.

Results: No significant differences in body, spleen or thymus
weights, although Cohn III-subfraction 5 apparently
induced an increase in thymus weights. Animals
examined days 7-9.

(d) Acute Expt. 20

Mice: C57Bl/6, 4 weeks old, female

Test substances: PBS, Cohn III-subfractions 1-14. Four
injections.

Results: All animals killed on day 7 after x-irradiation.
Thymus weights determined. Fraction 5 was highest,
but not significantly different from remainder.

(e) Acute Expt. 21

Mice: C57Bl/6, 4-5 weeks, male and female

Test substances: PBS, Cohn III-subfractions 5 and 13. Four
injections.

Results: Significant difference with Fr. 13 in wbc on 14th day. ($P < .001$). No significant differences in thymus weight with Fr. 13 and no significant differences in wbc or thymus weight with Fr. 5 between days 12 and 16.

Conclusions: Obviously, it has not been possible to pick up differences in subfractions using the very simple test systems of measurement of organ weights or wbc after a single dose of 300 r. The differences are obscured by individual variation, and large numbers of test animals would be required. As thymus DNA and RNA polymerase do reflect differences between whole serum and serum albumin or saline (C00-1391-18), an experiment like no. 20 above will have to be performed using the enzymes as test parameters. This is more time consuming and expensive, but apparently necessary.

(2) α_2 -Macroglobulin

An important aspect of the correlation between the effects of serum on the acute manifestations of radiation exposure and the long term, leukemogenic effect is the relationship of α_2 -macroglobulin (α_2M) to each of these effects. Berenblum and his associates think that α_2M possesses RLP activity (Nature 217:857, 1968). Nettesheim, Hanna and Fisher (Radiation Research 35:378, 1968) find that serum α_2M promotes regeneration of hematopoietic tissue when given shortly after irradiation. Because RLP activity was found in light serum fractions (C00-1391-10), we have considered the possibility mentioned above, that RLP is carried by α_2M . As part of our studies on the nature and effects of RLP, we have compared the behavior of α_2M

during the fractionation of spleen and serum. Part of this work will soon be published (COO-1391-18). Briefly, the trypsin esterase binding (TEBA) and the antitrypsin (TIC) activities of α_2M have been used to follow the protein during fractionation. Ammonium sulfate fractions of spleen and serum were eluted from Sephadex. A considerable portion of TEBA appears in the 40-60% ammonium sulfate fraction. TEBA of both spleen and serum follow the "19S" peak closely. Spleen TEBA elutes earlier than the serum activity, and TEBA represents a greater proportion of spleen extract total protein. TEBA and α_2M (as demonstrated immunoelectrophoretically) elute together. The spleen activity is more sensitive to heating at 60°, and is lost at pH 4.5. Both are fairly stable to pH 9.5. The TIC of serum is scattered over the Sephadex fractions, whereas that of spleen is confined to the 19S peak (Fig. 2). This behavior is reminiscent of that of RLP reported in COO-1391-10. Heating causes loss of spleen TEBA and TIC, whereas serum TEBA is stable and TIC little effected. TEBA of mouse serum decreases after 300 r and is restored by sheep serum but not saline or albumin. The effect of serum fractions, including α_2M , is under investigation.

These data show that there are differences in α_2M from different parts of the same animal (spleen and serum). Differences between human and mouse α_2M were shown in last year's progress report (COO-1391-17). These two proteins do not cross react in the Ouchterlony precipitin test, although on immunoelectrophoresis they migrate to almost the same place.

C. STUDENTS TRAINED

None this year

Lily C. Yip, Ph.D. Postdoctoral fellow, then staff 1965-69

Don B. Clewell Received Ph.D. 1967

Norman Blair Medical student

Darlene Sayers Medical student

Theresa Nong Graduate student

D. CONTEMPLATED CHANGE IN DIRECTION

We would like to continue both the search for RLP and the investigation of changes in biochemical parameters following x-irradiation and their reversal by serum or serum fractions. If this proves impractical because of financial limitations, we would prefer investigation of the reversal of the manifestations of "acute biochemical radiation sickness". This is more closely related to human problems, as radiation sickness and acute manifestations are more likely to follow accidental or therapeutic exposure than is the development of leukemia.

Table 1
Results obtained in the leukemia prevention
test with commercial Cohn fractions

Expt. No.	Material injected	Lymphosarcoma	P	Duration (days)
		Survivors at first lymphosarcoma		
84	Fr. IV, 0.5% PBS	8/33	.04	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	<.0001	365
109	α_2 -globulin, 1% PBS	3/44 29/44	<.0001	365
110	α_2 -globulin, 1% PBS	3/49 8/50	n.s.	365

Table 2

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

Expt. No.	Material injected	Lymphosarcoma	P	Duration (days)
		Survivors at first lymphosarcoma		
111	Fr. 1 PBS	*		1—
112	Fr. 2 PBS	*		—
113	Fr. 3 PBS	9/44 20/41	.008	365
114	Fr. 4 PBS	15/42 16/45	n.s.	365 365
115	Fr. 5 PBS	3/26 13/35	n.s.	365 365
116	Fr. 6 PBS	18/45 15/37	n.s.	365 365
117	Fr. 7 PBS	11/32 18/37	n.s.	365 365
118	Fr. 8 PBS	21/44 20/39	n.s.	365 365
122	Fr. 1 PBS	23/45 22/46	n.s.	365 365
123	Fr. 2 PBS	19/50 22/49	n.s.	365 365
124	Fr. 3 PBS	15/45 19/41	n.s.	365 365
125	Fr. 4 PBS	16/44 20/43	n.s.	365 365

Table 2 continued

126	Fr. 5 PBS	8/47 20/45	n.s.	365 365
127	Fr. 6 PBS	*		--
128	Fr. 7 PBS	4/49 1/47	n.s.	365 365
129	Fr. 8 PBS	7/46 10/42	n.s.	365 365
130	Fr. 9 PBS	1/47 6/40	n.s.	365 365
131	Fr. 10 PBS	*		--
132	Fr. 11 PBS	2/47 3/42	n.s. n.s.	365 365
133	Fr. 12 PBS	*		--
134	Fr. 13 PBS	*		--
135	Fr. 14 PBS	1/34 1/49	n.s.	365 365
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140	Fr. 1 PBS	11/48 14/47	n.s.	365
141	Fr. 2 PBS	*		--
142	Fr. 3 PBS	9/39 14/37	n.s.	365
143	Fr. 4 PBS	18/44 10/38	n.s.	365
144	Fr. 5 PBS	17/48 14/44	n.s.	365

Table 2 continued

145	Fr. 6 PBS	12/37 18/44	n.s. ¹	365
146	Fr. 7 PBS	13/41 23/41	n.s.	365
147	Fr. 8 PBS	*		--
148	Fr. 9 PBS	*		--
149	Fr. 10 PBS	11/50 13/43	n.s.	365
150	Fr. 11 PBS	21/48 23/45	n.s.	365
151	Fr. 12 PBS	6/46 24/43	.0002	365
152	Fr. 13 PBS	17/39 23/40	n.s.	365
153	Fr. 14 PBS	14/47 19/48	n.s.	365

* Terminated early owing to excessive mortality

¹ Borderline of significance

Table 3

Results obtained in the leukemia prevention test
with Cohn fraction IV chromatographed on a 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.	365
92	Fr. 5 PBS	9/32 12/32	n.s.	365
93	Fr. 6 PBS	7/31 18/33	n.s. ¹	365
94	Fr. 1 PBS	*		--
95	Fr. 2 PBS	11/43 22/47	n.s.	365
96	Fr. 3 PBS	13/46 17/49	n.s.	365
97	Fr. 4 PBS	*		--
98	Fr. 5 PBS	*		--
99	Fr. 6 PBS	*		--
100	Fr. 7 PBS	8/36 9/37	n.s.	365
101	Fr. 8 PBS	16/40 22/37	n.s.	365

* Terminated early owing to excessive mortality

¹ Borderline of significance

Table 4

Preliminary results obtained in the leukemia prevention test with Cohn IV-4 chromatographed on a DEAE-cellulose column

Expt. No.	Material injected	Lymphosarcoma	P	Duration (days)
		Survivors at first lymphosarcoma		
154	Fr. 1 PBS	19/41 18/41	n.s.	365
155	Fr. 2 PBS	17/44 23/47	n.s.	365
156	Fr. 3 PBS	20/39 15/40	n.s.	365
157	Fr. 4 PBS	15/40 32/46	n.s.	354
158	Fr. 5 PBS	16/49 15/49	n.s.	354
159	Fr. 6 PBS	19/39 19/41	n.s.	341
160	Fr. 7 PBS	18/26 21/29	n.s.	341
161	Fr. 8 PBS	17/43 16/36	n.s.	326
162	Fr. 9 PBS	11/28 25/31	n.s.	326
163	Fr. 10 PBS	21/37 23/42	n.s.	315
164	Fr. 11 PBS	19/47 25/49	n.s.	315
165	Fr. 12 PBS	17/44 25/43	n.s.	305
166	Fr. 13 PBS	14/47 28/45	.05	305
167	Fr. 14 PBS	8/47 17/43	n.s.	285

Table 5

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

Expt. No.	Material injected ¹	Lymphosarcoma	P	Duration (days)
		Survivors at first lymphosarcoma		
74	Hgb, 0.05% PBS	4/22 31/40	<.0001	365
86	Hgb, 0.05% PBS	16/37 14/42	n.s.	365
102	Hgb, 0.05% PBS	9/36 17/31	n.s.	365
103	Hgb, 0.025% PBS	16/46 25/43	n.s.	365
104	Denat. hgb, 0.025% PBS	11/44 16/45	n.s.	365
105	Hemin, 0.025% PBS	5/46 20/39	n.s.	365
106	Globin, 0.025% PBS	11/42 14/44	n.s.	365
108	Globin, 0.05% PBS	6/36 13/45	n.s.	365
137	Hemin, 0.1% PBS	15/44 21/46	n.s.	365
139	Hemoglobin, 0.5% PBS	12/34 16/43	n.s.	365

¹ 50 mice entered in each test

Table 6

Miscellaneous experiments and materials entered in
leukemia prevention tests of less than 8 month's duration

Expt. No.	Material injected ¹	Lymphosarcoma	P	Duration (days)
		Survivors at first lymphosarcoma		
120	Mouse tissue extract PBS	18/48 30/47	n.s.	365
121	Mouse tissue extract PBS	27/45 24/44	n.s.	365
136	Sheep globulin, 0.1% PBS	*		--
138	Fibrinogen, 0.5% PBS	16/28 17/40	n.s.	365
181-193	Sheep Cohn IV-4 on DEAE			
194-206	Sheep Cohn IV-4 on DEAE			

¹ 50 mice entered in each test

* Terminated early owing to excessive mortality

Fig. 1. Fractionation of sheep serum commercial Cohn fraction IV-4 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).

Fig. 2. Distribution of antitrypsin activity and trypsin-protein esterase binding activity after gel filtration on a 1.2 x 72 cm Sephadex G-200 column of an ammonium sulfate precipitate (40-60% saturation) of sheep serum. Fraction size, 5 ml. Sample size, 2 ml. Both activities are on the ordinate expressed as absorbancy at 410 m μ (ΔA_{410}) per min per ml. Fractions are indicated along the abscissa. The thin continuous line indicates the absorption at 280 m μ , the heavy continuous line the TPEBA, and the dotted line the TIC.

Figure 2