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PROGRESS REPORT

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

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Principal Investigator: M. E. Hodes, M.D., Ph.D.
Professor of Medicine and Biochemistry

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SUMMARY:

This report covers the entire grant period to date (2 1/2 years). During this time the following has been accomplished: (1) Confirmation of the original observations of Berenblum, Cividalli, Trainin and Hodes (Nature 202:973, 1964; Blood 26:8, 1965) that 40% aqueous sheep spleen extracts contain a factor (RLP= radiation leukemia protection) that decreases the incidence of leukemia in x-irradiated C57BL/6 mice, when injected after exposure to x-ray. (2) Extension of the finding to BALB/C mice (3) Notation of the toxic effect (anaphylaxis) of RLP in x-irradiated C3H/eb mice and probable lack of protective effect in RF mice. (4) Demonstration of RLP effect after injection of 10% (as well as 40%) extracts. (5) Demonstration of RLP effect after injection of cow spleen extracts (and of certain Cohn fractions from cow and sheep). (6) Demonstration that RLP-containing extracts of sheep spleen do not protect mice against the lethal effect of 550-800 r of x-irradiation. (6) The finding that RLP is present in sheep (and cow) serum, and in the precipitate resulting from addition of ammonium sulfate to sheep serum. (7) Serum albumin has been shown to be devoid of RLP activity, and commercial serum γ -globulin contains only trace amounts. Comparison of eluates from Sephadex columns by gel electrophoresis has helped localize RLP to the α - or β -globulin fraction of serum, and shown it to be precipitable between 0 and 40% saturation with ammonium sulfate, and to elute early from Sephadex G-200. (8) A series of experiments has shown RLP to be stable to mild, brief heating and to freeze-drying, to be unable to prevent the rise in spleen

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IN BIOLOGICAL SCIENCE ABSTRACTS

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DNase II seen after sublethal doses of x-ray and to be devoid of salutary effect on the chromosome damage evoked by x-ray in cultures of Chinese hamster cells.

In brief, RLP has been ^{partially} localized to the early eluting fraction obtained after filtration on Sephadex G-200 of the precipitate obtained after 40% saturation of spleen extract or serum with ammonium sulfate. By comparison of gel electrophoresis patterns of the eluates and their activities in the mouse anti leukemia assay, RLP has been shown to travel in the α - β region. Studies on the mode of action of RLP have in part ruled out several possible mechanisms, but have not yet demonstrated how the material exerts its protective action.

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7. COO-1391-7 M. E. Hodes and D. B. Clewell. Prevention of x-ray induced leukemia in mice: Fractionation of sheep spleen extracts and search for RLP. Abstracts IXth International Cancer Congress, Tokyo, 1966.
8. COO-1391-8 M. E. Hodes and D. B. Clewell. Progress in isolation of a factor preventing x-ray induced leukemia. (Abstract) Fed. Proc., Submitted.
9. COO-1391-9 Progress Report January 31, 1966

The remainder of this progress report is arranged as restatements and answers to the questions asked by Dr. James L. Murray in his letter of 25 April 1966.

(1) Main research accomplishments, with special reference to originally stated objectives.

(a) The original objective of isolation of RLP has been partially attained.

Both sheep spleen and serum have been fractionated with ammonium sulfate, and RLP localized to two Sephadex peaks. After fractionation of the once amm. sulf. precipitated material on Sephadex G-200 RLP is localized to crude Peak 1 (0-40%) and crude Peak 3(40-60%) [Table X]. Comparison of column eluates of reprecipitated amm. sulf. fraction and Cohn fractions by electrophoresis in polyacrylamide gel together with the results of leukemia prevention experiments suggest that RLP travels with α or β globulin, but is probably not an albumin or γ -globulin.

(b) RLP has been shown to be stable to lyophilization and brief heat treatment (60°, 10 min).

(c) RLP activity has been found in bovine spleen and serum as well as in sheep serum. Because serum provides a convenient "clean" source, it is now used as a starting material for fractionation experiments.

(d) BALB/c, C3H/eb and RF/ mice were used in leukemogenesis experiments.

There was a definite decrease in leukemia incidence in treated BALB/c

mice. The C3H/eb mice suffered from anaphylactic shock after radiation and injection, a phenomenon not seen in controls or other strains. The treated RF mice (a small group) had as many myelogenous-type leukemias and lymphosarcomas as their controls.

(e) RLP-containing extracts of sheep spleen did not prevent death from acute exposure to 550-800 r in several strains of mouse, thus clearly differentiating it from the various antilethal factors described in the literature.

This project was designed to isolate RLP, the Radiation-Leukemia Protective factor, from sheep spleen. The work was based on the observation of Berenblum, Cividalli, Trainin and Hodes (Blood 26:8, 1965) that 40% aqueous extracts of sheep spleen contain a factor (RLP) that prevents the leukemia seen after 5-12 months in mice irradiated with 180 r once weekly for 4 weeks when multiple (biweekly to weekly) injections are given after exposure to x-ray. This observation has been confirmed by us (Hodes, Clewell, Hubbard & Yu, Cancer Research 26:1780, 1966, Bibliography, Reference C00-1391-5) for both male and female mice. We have used a 13 month observation period. Table 1, taken from this paper and reproduced below, shows that 10% as well as 40% extracts are effective.

TABLE 1
INCIDENCE OF LYMPHOSARCOMA IN C57BL/6 MICE TREATED WITH SHEEP SPLEEN EXTRACTS

Experiment ^a	Treatment	Sex	No. of lympho- sarcomas	Lymphosarcoma inci- dence at 400 days, corrected ($\pm$ S.E.)	Difference from control	P	
A. 40% extracts	SSE-1	Control	M	16	0.739 $\pm$ 0.222	0.431	0.018
		Spleen	F	4	0.308 $\pm$ 0.188		
	Control	F	19	0.735 $\pm$ 0.190	0.472		
		Spleen	M + F	4		0.263 $\pm$ 0.157	
					0.452		
		SSE-2	Control	M	15	0.663 $\pm$ 0.168	0.632
	Spleen		F	1	0.031 $\pm$ 0.031		
	Control	F	17	0.739 $\pm$ 0.180	0.323		
Spleen		M + F	9	0.416 $\pm$ 0.134			
				0.478			
	B. 10% extracts	SSE-3	Control	M	17	0.779 $\pm$ 0.216	0.529
Spleen			F	6	0.250 $\pm$ 0.105		
Control		F	23	0.900 $\pm$ 0.177	0.400		
		Spleen	M + F	16		0.500 $\pm$ 0.132	
					0.464		
		SSE-4	Control	M	8	0.812 $\pm$ 0.332	0.512
Spleen			F	3	0.300 $\pm$ 0.168		
Control		F	10	0.497 $\pm$ 0.169	0.248		
	Spleen	M + F	5	0.249 $\pm$ 0.117			
				0.350			

^a N = 50.

With confirmation of the finding of RLP activity in spleen, attempts were made to find a better test system. To this end BALB/c and RF mice were tried. As is obvious from Table 2 (of Reference COO-1391-5) the overall incidence of leukemia (= thymic lymphosarcoma) in BALB/c is lower than in C57BL/6 (Table 1), but RLP offers significant

TABLE 2
INCIDENCE OF LYMPHOSARCOMA IN BALB/c AND RF MICE TREATED WITH SHEEP
SPLEEN EXTRACTS

Strain	Treatment	Sex	N	No. of lympho- sarcomas	Lymphosarcoma inci- dence at 400 days, corrected ($\pm$ S.E.)	Difference from control	P
BALB/C	Control	M	23	5	0.342 $\pm$ 0.160	0.342	0.001
	40% spleen		24	0	0.0		
	Control	F	21	0	0.338 $\pm$ 0.128	0.338	
	40% spleen		21	0	0.0		
		M + F				0.340	
	Control	M	26	10	0.558 $\pm$ 0.157	0.384	
	10% spleen		27	4	0.174 $\pm$ 0.083		
	Control	F	24	6	0.279 $\pm$ 0.104	0.235	
	10% spleen		23	1	0.044 $\pm$ 0.043		
		M + F				0.310	
RF	Control	M	8	1		0.003	
	40% spleen		19	4	0.677 $\pm$ 0.301		
	Control	F	19	12	0.859 $\pm$ 0.203		
	40% spleen		24	14	0.947 $\pm$ 0.222		

protection from leukemia never-the-less. No protection was seen in the small group of RF mice. Many of the C3H/eb mice died, apparently from anaphylactic shock, after the 4th injection of RLP. This result is shown in Chart 2 from Reference COO-1391-5.

CHART 2. Deaths in C3H/eb mice following X-irradiation (180 r X 4) and injection of sheep spleen extract or saline solution. Two separate experiments and their controls are shown. Because of the high mortality, these experiments were discontinued shortly after inception, and well before leukemia could develop.

A number of other attempts have been made to develop a shorter assay for RLP. These experiments of necessity bear on the mechanism of action of RLP.

(a) Attempts to demonstrate an antilethal action of RLP. Three strains of mice were exposed to single doses of 550-800 r. The mice were injected with RLP either before or after radiation. As is evident from Table 3 (from Reference COO-1391-5)

TABLE 3
DEMONSTRATION OF THE FAILURE OF RLP TO PROTECT
AGAINST LETHAL ACTION OF X-RAY

Experiment*	X-ray dose (r)	Sex	N	Fraction dead at 30 days	Difference from control	P
A. Swiss						
1. cont.	550	F	31	0.677		
pre-1			29	0.448	0.129	n.s.
post-3			30	0.400	0.277	0.05
2. cont.	550	F	50	0.480		
post-1			50	0.300	0.180	<0.05
3. cont.	550	F	30	0.467		
post-1			30	0.767		
post-5			30	0.467	0	n.s.
4. cont.	550	F	30	0.733		
post-1			30	0.867		
post-5			30	0.800		
5. cont.	700	F	30	0.967		
pre-1			30	0.967	0	n.s.
post-1			30	1.000		
6. cont.	700	F	48	0.875		
pre-1			100	0.900		
7. cont.	800	F	30	1.000		
pre-1			30	1.000	0	n.s.
post-10			30	0.967	0.033	n.s.
B. C57BL/6						
1. cont.	550	M + F	30	0.400		
post-1			30	0.000	0.400	0.005
2. cont.	700	F	30	0.967		
pre-1			30	0.933	0.034	n.s.
post-10			30	0.967	0	n.s.
3. cont.	700	F	30	1.000		
pre-1			30	0.933	0.067	n.s.
post-1			30	0.900	0.100	n.s.
4. cont.	700	F	50	0.980		
post-1			50	1.000		
5. cont.	550	F	25	0.720		
post-1			25	0.880		
6. cont.	550	M	25	0.880		
post-1			25	0.760	0.120	n.s.
7. cont.	550	M	20	0.300		
post-1			20	0.400		
C. C3H/eb						
1. cont.	550	M	20	0.333		
post-1			20	0.367		
2. cont.	550	M	20	0.150		
post-1			20	0.450		
3. cont.	800	F	30	1.000		
post-1			30	0.967	0.033	n.s.
4. cont.	800	F	50	1.000		
post-1			50	1.000	0	n.s.

* Cont. = control; pre-1 = 1 injection prior to X-ray; post-3 = 3 injections after X-ray; n.s. = not significant at the 5% level.

* 1-4 = C57BL/6; 5-7 = C57BL/10.

no schedule of injections produced consistent decreases in 30 day mortality.

(b) Decrease in elevation of spleen and liver DNase II after exposure to a single dose of 800 r. This was reported in C00-1391-4 (Ref. 4). The conclusion of

these experiments was: Forty-eight hours after whole body x-irradiation with 800 r, there is a marked increase in DNase II activity of spleen, but not liver. This increase is apparently not retarded by administration of RLP either before or after x-irradiation.

(c) Action of RLP on irradiated tissue cultures.

The Chinese hamster derived cell line, CH461-T71-17, a gift of Dr. Chu, were used in these experiments. Fifty-six small Petri dishes were seeded with 1.5 ml. of a culture containing 100,000 cells/ml, fed with Puck's N16 reg. medium containing 15% fetal calf serum, and grown in a CO₂ incubator for 48 hours. Forty dishes were irradiated with 400 r, after which 12 were treated with RLP in a final concentration of 5%, 12 with RLP in a final concentration of 1%, and 16 received fresh medium. The cells were fixed and stained at 8, 12, 25 and 30 hours post irradiation. There was no difference in chromosomal aberrations between treated and untreated cells.

(d) Action of RLP on mouse bone marrow.

C57BL/6 mice were given the usual leukemogenic dose of x-ray. A total of 140 animals, 70 of each sex were used. Of these, half were experimental, and half control animals. Two males and 2 females were colchicized and sacrificed on days 2, 3 and 7 after the irradiation each week. Marrow was removed from the femurs treated with hypotonic citrate and fixed. At least one slide, and often more, from each mouse was scanned for mitoses. There was little difference in mitotic index between RLP treated and untreated animals (see Fig.1)

Fig. 1

This experiment has been extended to mice sacrificed later (at a time when leukemias are expected) than the group reported here. Results of this experiment are forthcoming.

(e) Action of RLP on mice injected with the Rauscher virus.

A small group of mice were used to test the action of RLP in preventing Rauscher leukemia. The results of this experiment are shown in Table 4 (from Reference C00-1391-6). Obviously, RLP offers no protection against the virus.

TABLE 4

FAILURE OF SHEEP SPLEEN EXTRACTS TO PROTECT
AGAINST RAUSCHER LEUKEMIA

Sex	Treatment	N	Leukemia incidence at 50 days	P
M	Control	8	0.875	n.s.*
	Spleen	8	1.000	
F	Control	8	0.625	n.s.
	Spleen	8	0.875	

* n.s., not significant.

The search for RLP itself has necessitated a number of preliminary experiments designed to test the nature and stability of RLP. These were followed by development of a fractionation scheme for spleen, and more recently serum. As a year must elapse between each experiment and its final evaluation, modifications have to be introduced with caution.

The results of a number of the characterization experiments are shown below:

TABLE 5

RLP activity of miscellaneous fractions

Experimental	Treatment	Sex	N	No. of lympho sarcomas	Corrected Incidence + S.E.	Difference from control	P
30% Ammon. Sulf. fraction from Spleen	Control	M	50	17	0.461 $\pm$ 0.108		
	Exp.		55	12	0.329 $\pm$ 0.090	0.132	
	Control	F	55	19	0.481 $\pm$ 0.106		
	Exp.		50	9	0.220 $\pm$ 0.071	0.261	
		M + F					0.02
30% A.S. Passed through DEAE Cell. at pH 6.0	Control	M	52	31	0.749 $\pm$ 0.121		
	Exp.		49	21	0.541 $\pm$ 0.108	0.208	
	Control	F	52	28	0.746 $\pm$ 0.131		
	Exp.		50	29	0.768 $\pm$ 0.120	--	
		M + F					n.s.
Spleen extract with mild heat treatment	Control	M	50	22	0.523 $\pm$ 0.106		
	Exp.		50	4	0.087 $\pm$ 0.050	0.436	
	Control	F	47	24	0.665 $\pm$ 0.122		
	Exp.		49	9	0.218 $\pm$ 0.070	0.447	
		M + F					0.0001
Spleen extract Lyophilized	Control	M	47	21	0.498 $\pm$ 0.092		
	Exp.		48	11	0.297 $\pm$ 0.080	0.201	
	Control	F	50	28	0.709 $\pm$ 0.078		
	Exp.		50	12	0.291 $\pm$ 0.081	0.418	
		M + F					0.0001
40% Ammon. Sulf. Fraction of Sheep Serum	Control	M	28	7	0.465 $\pm$ 0.164		
	Exp.		25	1	0.059 $\pm$ 0.058	0.406	
	Control	F	27	14	0.518 $\pm$ 0.096		
	Exp.		25	1	0.048 $\pm$ 0.047	0.470	
		M + F					<0.00001

In addition to these results on sheep fractions, results summarized in Table 6 (from Reference C00-1391-5) show activity in bovine spleen extracts and bovine gamma globulin.

TABLE 6
INCIDENCE OF LYMPHOSARCOMA IN C57BL/6 MICE TREATED WITH BOVINE SPLEEN
EXTRACTS OR PROTEINS

Experiment	Treatment	Sex	N	No. of lympho-sarcomas	Lymphosarcoma incidence at 400 days, corrected ($\pm$ S.E.)	Difference from control	P
BSE-1 ^a	Control	M	46	14	0.657 $\pm$ 0.170	0.407	0.004
	Spleen		46	7	0.250 $\pm$ 0.087		
	Control	F	34	13	0.489 $\pm$ 0.122	0.302	
	Spleen		35	4	0.187 $\pm$ 0.093		
		M + F				0.355	
BALb-1 ^b	Control	M	50	23	0.610 $\pm$ 0.112	0.029	n.s. ^c
	Albumin		50	19	0.587 $\pm$ 0.126		
	Control	F	50	17	0.624 $\pm$ 0.154	0.204	
	Albumin		50	17	0.420 $\pm$ 0.097		
		M + F				0.117	
BGlob-1 ^d	Control	M	52	17	0.564 $\pm$ 0.123	0.169	0.018
	Globulin		50	8	0.395 $\pm$ 0.137		
	Control	F	49	20	0.606 $\pm$ 0.144	0.422	
	Globulin		49	6	0.244 $\pm$ 0.083		
		M + F				0.296	

^a Bovine spleen extract, 40%.

^b Bovine serum albumin.

^c Not significant.

^d Bovine γ -globulin.

The bovine gamma globulin was injected with alum as adjuvant. Later experiments, shown in Table 7, show bovine α -globulin without alum to be inactive.

TABLE 7
RLP activity of Cohn fractions II and V

Experimental	Treatment	Sex	N	No. of lympho sarcomas	Corrected Incidence + S.E.	Difference from control	P
40% Bovine Cohn Fraction V (Albumin)	Control	M	50	23	0.616 $\pm$ 0.112	0.027	n.s.
	Exp.		50	19	0.587 $\pm$ 0.126		
	Control	F	50	17	0.624 $\pm$ 0.154		
	Exp.		50	17	0.420 $\pm$ 0.097		
	M + F						
1% Bovine Cohn Fraction V (Albumin) with .um	Control	M	50	12	0.271 $\pm$ 0.073	0.092	n.s.
	Exp.		50	3	0.179 $\pm$ 0.102		

Table 7 Continued

4% Bovine	Control	F	50	38	0.833 ± 0.113		
Cohn Fraction II	Exp.		50	28	0.668 ± 0.105		
						0.165	n.s.
1% Bovine	Control	M	40	14	0.584 ± 0.140		
Cohn Fraction II	Exp.		35	15	0.416 ± 0.103	0.168	
	Control	F	35	12	0.365 ± 0.099		
	Exp.		40	12	0.414 ± 0.113	--	
		M + F					n.s.

This suggests RLP is a contaminant of the γ -globulin fraction, a conclusion borne out by other work.

Fractionation of Sheep Spleen and Serum.

After demonstration of RLP activity in ammonium sulfate precipitates of sheep spleen, attempts were made to separate RLP from the dissolved precipitates on Sephadex G-200 columns. These experiments led to collection of several peaks (fractions) which were subsequently tested for antileukemic activity. The results of the antileukemia tests are shown below: (Diagrams on p. 12a)

TABLE 8

A. 0-40% Ammonium Sulfate							
Peak 1	Control	M	25	14	0.622 ± 0.145		
	Exp.		25	1	0.056 ± 0.054	0.566	
	Control	F	30	17	0.680 ± 0.109		
	Exp.		25	7	0.327 ± 0.117	0.353	
		M + F					0.00002
Peak 2	Control	M	25	11	0.477 ± 0.114		
	Exp.		25	4	0.167 ± 0.076	0.310	
	Control	F	25	13	0.523 ± 0.109		
	Exp.		25	15	0.644 ± 0.139	--	
		M + F					n.s.
Peak 3	Control	M	25	9	0.366 ± 0.107		
	Exp.		25	5	0.202 ± 0.084	0.164	
	Control	F	25	15	0.625 ± 0.102		
	Exp.		25	13	0.520 ± 0.100	0.105	
		M + F	25				n.s.
B. 40-60% Ammonium Sulfate							
Peak 1	Control	M	25	9	0.391 ± 0.104		
	Exp.		25	4	0.184 ± 0.087	0.207	
	Control	F	25	8	0.358 ± 0.111		
	Exp.		25	16	0.667 ± 0.100	--	
		M + F					n.s.
Peak 2	Control	M	25	3	0.130 ± 0.071		
	Exp.		25	9	0.398 ± 0.120	--	
	Control	F	25	11	0.543 ± 0.144		
	Exp.		25	4	0.174 ± 0.080	0.369	
		M + F					n.s.

Peak 3	Control	M	25	6	0.255 ± 0.097		
	Exp.		25	11	0.048 ± 0.047	0.207	
	Control	F	25	5	0.357 ± 0.143		
	Exp.		25	6	0.261 ± 0.093	0.096	
		M + F					0.05
Peak 4	Control	M	25	5	0.216 ± 0.092		
	Exp.		25	10	0.479 ± 0.132	--	
	Control	F	25	6	0.292 ± 0.104		
	Exp.		25	10	0.603 ± 0.164	--	
		M + F					n.s.

This fractionation was subsequently improved by reprecipitation of the ammonium sulfate fractions three times, with elimination of much of the overlap between fractions (as determined by electrophoresis at pH 8.2 in polyacrylamide gel).

With the demonstration of activity in (sheep) serum, this material was also fractionated by a combination of amm. sulf. precipitation and Sephadex filtration, with control by gel electrophoresis. (The serum fractions are currently undergoing test in mice). Comparisons have been made between the spleen and serum fractionations, in an attempt to eliminate some proteins from consideration and to determine which of the others are likely candidates for RLP. A comparison of the Sephadex fractionations of 0-40% saturated ammonium sulfate precipitates of spleen and serum are shown below:

Fractionation on G-200 Sephadex (3.5 x 100 cm) of twice reprecipitated Ammon. Sulf. 0-40% fractions of (A) spleen and (B) serum of sheep.

Gel electrophoresis (p. 15) of the pools indicated show certain similarities. Similar bands are seen at level 7 in both Spl and Ser. This band is even more prominent in Se2, and absent from Sp2. Experiments still in the early stages indicate little activity in Peak 1 of the figure, thus probably ruling out the level 7 band as RLP. A band at level 3 runs through all fractions except Spl. Bands at levels 3 and 3 1/2 are also seen in Cohn Fraction II, and the band at level 7 is present in Cohn Fraction III.

Immunoelectrophoretic studies suggest a β - to γ -globulin similarity between Sp2 and Se2 and that 7S γ -globulin is common to Sp3 and Se3. These fractions are under test in mice.

If RLP is present in (mouse) serum, one may suppose that it is decreased or altered after x-irradiation of the mice. We have begun comparing (by gel electrophoresis) the sera of unirradiated, irradiated and irradiated + RLP treated mice. Such a comparison is shown below:

Serum from C57BL/6 mice 7 days after
last x-ray.

A = irradiation + PBS

B = irradiation + RLP

C = non irradiated age controls

This study is complicated by the differences in patterns seen in male and female mice, and by developmental differences in the young (30 day old) mice used in the experiments. Nevertheless, some differences are seen near the bottom of the gels. These are being explored.

The studies so far eliminate albumin as RLP. γ -globulin, shown to possess RLP activity in the presence of alum, is inactive when injected alone. The commercial fraction used in the original experiments is indeed electrophoretically heterogeneous, although it is about 95% 7S γ -globulin. We can localize 7S to peak 3 on the serum fractionation diagram above. If RLP is present in this peak, it is as a very minor constituent. Comparison of the electrophoretic patterns of these peaks leads us to the conclusion that RLP travels under the influence of an electric current with the α or β globulins.

(2) Plans for continuation of present objectives and possible new objectives in consideration of past results.

The fractionation of fresh sheep serum and of commercial Cohn fractions prepared from sheep serum is continuing. The techniques of ammonium sulfate precipitation and Sephadex column fractionation monitored by polyacrylamide gel electrophoresis are being exploited. These fundamental techniques are also being improved in the course of the work. While the studies to date have localized RLP to two peaks, these are scattered in the 0-40% and 40-60% amm. sulf. precipitates of sheep serum.

Sephadex filtration fractions will be separated into acidic and basic proteins. This will be accomplished by passage through columns of DEAE-cellulose and CM-cellulose. The material passing through ("pass through") will be separated from the material sticking to the column. The latter will be eluted with strong salt and pH change, providing us with 2 fractions from each column, or 4 fractions from each Sephadex peak. This is an extension and modification of work envisioned in the original application, and which led to the result shown in Table 5 above.

Use is being made of the LKB ReCychrom to sharpen and refine the Sephadex fractionation. As RLP is separated from the bulk of the other proteins it will be possible to make greater use of this and other techniques designed for smaller amounts of protein. The current approach is to prepare large amounts of material for injection by ammonium sulfate precipitation and column chromatography, checking each fraction for overlaps with its neighbors by gel electrophoresis. As the results of the mouse antileukemia assays become available, certain peaks and bands can be ruled out of consideration, and means sought for separating those proteins which remain likely candidates for RLP.

Our results have suggested several modifications in procedure. First, we have shortened the period of observation from 13 to 12 months. This provides a saving

in space and man-hours required for mouse care, at no loss in statistical significance of the results. Second, we are using smaller groups of mice, but running many of the experiments in duplicate. Third, we have continued the experiments with C57Bl/6 and terminated work with other strains, for reasons apparent from the test results, as well as from economic considerations. Also, mice are now purchased entirely from a local breeder, our own breeding colony having been turned over to him for care and production. Fourth, mouse care, both routine and special, is entirely under our supervision, providing us with more intimate control over experimental and control animals. Fifth, we shall probably be able to eliminate the 40-60% amm. sulf. fractions in the near future.

Communication has been and will be maintained with Dr. I. Berenblum's group at the Weizmann Institute, Rehovoth, Israel. This group has found RLP activity in human serum, and is using its own fractionation scheme to separate the material from this source. They believe RLP may be an α -globulin (Abst. IXth International Cancer Congress, Tokyo, 1966), a result not incompatible with our findings.

The experiments on chromosome repair and DNase activity, and investigation of other enzymes will be continued as time permits. It is hoped that these, and the gel electrophoresis studies on the irradiated mice, will furnish information on the mode of action of RLP.

(3) Graduate students trained.

Mr. Don B. Clewell has been trained as a graduate student in the Department of Biochemistry. He will receive his Ph.D. in June, 1967 on a thesis entitled "*Studies on the induction and prevention of radiation induced leukemia in mice*", after which he will work as a postdoctoral fellow with Dr. D. R. Helinski at the University of California, San Diego, California.

(4) Bibliography: Presented at beginning (p. 2) of this report. In addition, we are preparing a manuscript on the fractionation of sheep spleen and serum. This will be submitted to Cancer Research or the International Journal of Cancer.

(5) Opinion on present state of knowledge in this area, its significance in the fields of biology and medicine, and needed future investigations.

Unfortunately, the present and meager state of knowledge in this field has already been summarized in the brief introductory statement to this grant. The only other worker in the field using subcellular materials for leukemia prevention is Berenblum, in whose laboratories the initial observations on RLP were made, and with whom the principal investigator worked. Kaplan and Brown (Cancer Res. 12:445, 1952 and Wasi and Black (Cancer Res. 21:463, 1961) were able to inhibit leukemia development with testosterone, but this steroid hormone is not likely to be RLP, nor is it appealing as a nontoxic substance to be administered to females as well as males.

The significance of a material which can be administered in small amounts after exposure to ionizing radiation and which will prevent leukemia is obvious in these days of therapeutic and diagnostic x-ray, hazard from atomic accident, and almost certain space travel. Berenblum's observation that human serum contains RLP makes the prospect of development of such an agent even more promising.

Future investigation, after isolation of RLP, will be concerned with search for the proper conditions under which the discovery can be exploited in human beings.

(6) Present division of federal support for our overall research program.

Research Grants:

(1) The structure of nucleic acids from human tissues, ¹USP.S grant No. 5R01 CA-03475-10.
\$10,800 (Renewal requested in amount of \$23,627 for next year)

1 September 1966-31 August 1967.

(2) Biochemical studies on the Shope rabbit papilloma

USPHS Grant No. CA-08120-03

\$43,902

1 January 1967-31 December 1967

Training Grants:

USPHS Grant No. 5 R01 CA-05066-07

This provides part of Dr. Hodes' salary and graduate student stipends for Mr. Clewell and one other student.

CONTRACTOR'S RECOMMENDATION FOR DISPOSITION OF RESEARCH DOCUMENT

(See Instructions on Reverse Side)

TITLE OF DOCUMENT

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

2. PERSONAL AUTHOR(S)

M. E. Hodes, M.D., Ph.D.

3. CONTRACTOR'S NAME AND ADDRESS

Indiana University Medical Center
1100 W. Michigan Street
Indpls., Ind.

4. CONTRACT NUMBER

AT (11-1)-1391

5. DATE OF DOCUMENT

1-6-64 thru 31-1-66

6. REPORT NUMBER

COO-1391-9

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ORGANIZATION

Indiana University Medical Center

TITLE

Professor of Medicine and Biochemistry

DATE

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M. E. Hodes

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INDIANA UNIVERSITY
CONTRACT No. AT(11-1)-1391
PROGRESS REPORT

UNITED STATES ATOMIC ENERGY COMMISSION

"ISOLATION OF A FACTOR PREVENTING X-RAY INDUCED LEUKEMIA"Period Covered: 1 June 1964 - 31 January 1966Principal Investigator: M. E. Hodes, M.D., Ph.D.
Professor of Medicine and BiochemistrySUMMARY:

This report covers the entire grant period to date (2 1/2 years). During this time the following has been accomplished: (1) Confirmation of the original observations of Berenblum, Cividalli, Trainin and Hodes (Nature 202:973, 1964; Blood 26:8, 1965) that 40% aqueous sheep spleen extracts contain a factor (RLP= radiation leukemia protection) that decreases the incidence of leukemia in x-irradiated C57BL/6 mice, when injected after exposure to x-ray. (2) Extension of the finding to BALB/C mice (3) Notation of the toxic effect (anaphylaxis) of RLP in x-irradiated C3H/eb mice and probable lack of protective effect in RF mice. (4) Demonstration of RLP effect after injection of 10% (as well as 40%) extracts. (5) Demonstration of RLP effect after injection of cow spleen extracts (and of certain Cohn fractions from cow and sheep). (6) Demonstration that RLP-containing extracts of sheep spleen do not protect mice against the lethal effect of 550-800 r of x-irradiation. (6) The finding that RLP is present in sheep (and cow) serum, and in the precipitate resulting from addition of ammonium sulfate to sheep serum. (7) Serum albumin has been shown to be devoid of RLP activity, and commercial serum γ -globulin contains only tract amounts. Comparison of eluates from Sephadex columns by gel electrophoresis has helped localize RLP to the α - or β -globulin fraction of serum, and shown it to be precipitable between 0 and 40% saturation with ammonium sulfate, and to elute early from Sephadex G-200. (8) A series of experiments has shown RLP to be stable to mild, brief heating and to freeze-drying, to be unable to prevent the rise in spleen

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