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Immunogenicity and Paralytogenicity of Antigens Solubilized from Line I_b Malignant Lymphocytes[1] (40048)

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When young C58 mice (<6 month-old) are immunized (1) to syngeneic line I_b malignant lymphoid cells (I_b cells) they are immune to a challenge to 10^7 viable cells. Immunity persists for at least a year (1) and is primarily T cell mediated (2). However, when young immunosuppressed C58 mice (3), or those immunologically compromised by age (4), are similarly immunized they develop a paralytic central nervous system disease (immune polioencephalomyelitis or IPE) that is characterized by an inflammatory destruction of neurons in the cord and brain stem (5). Current evidence indicates that as C58 mice age they spontaneously lose a T cell subpopulation resistant to 350R (6) that protects them from induction of the disease. We are interested in the central question of whether the I_b cell antigen that immunizes C58 mice to transplantable leukemia is also the one that elicits IPE. As a first step we compared the relative effectiveness of low energy sonication and 3 *M* KCl extraction for preparing solubilized antigens. We report here that antigens solubilized from I_b cells by either method elicited IPE in indicator C58 mice and immunized immunocompetent animals to the disease. Crude solubilized preparations also immunized mice to transplanted line I_b leukemia.

Methods. C58 mice. The linage of our strain of C58 mice (C58/wm) and the methods of mouse husbandry were described (1, 7). X-Irradiated 5 month-old, or non-x-irradiated 9–12 month-old mice, were used for assays of IPE (3). *I_b cells.* The methods used to prepare suspensions of viable I_b cells were described (1, 2). Spleens removed from mice moribund from transplanted leukemia were dispersed gently in cold (4°) HBSS (Hanks balanced salt solution) and teased apart with scalpels to yield single cell suspensions. Cell preparations were allowed to stand 3–5 min to let gross particles settle. The supernatant cells were transferred to a conical centrifuge tube and sedimented at 250*g* for 3 min. The sedimented cells were washed three times and resuspended in 10 ml of HBSS. Total and viable cell counts (trypan blue exclusion) were done as described previously (1, 2). Unless specified otherwise suspensions contained *ca.* 10^8 viable cells/ml.

X-Irradiation. X-Irradiated I_b cells were used to elicit IPE (3). I_b cells were irradiated in a 60 × 15 mm plastic dish containing 5–10 ml of cell suspension. Cells were irradiated by a Westinghouse Coronado X-ray Therapy Machine operated at 200 KV and 15 ma through a 1.0 mm Al filter to produce a dose of 527.5 R/min at a target distance of 24 cm. A dose of 10,000 R was used. Five month-old C58 mice used for IPE assays received 525 R with the machine calibrated to deliver 67 R/min at a target distance of 70 cm when operated at 250 KV and 15 ma using 0.5 mm Cu and 1.0 mm Al filters. One day after x-irradiation they were challenged by the ip injection of 10^2 or 10^3 x-irradiated I_b cells.

Assays for IPE. The main clinical and histopathologic features of the disease and the statistical limits of assays, were described (3). The earliest onset occurs 9–10 days after the ip injection of 10^3 x-irradiated I_b cells with a peak at 12–15 days. Since IPE may develop as late as 20–30 days all mice were scored over a 30-day observation period.

Sonication. Care was taken to employ the optimum conditions described by Reisfeld and Kahan (8). Viable cells were sonicated for 5 min (10 KHz @ a.25 ma, 4°) using a Raytheon low intensity DF101 oscillator.

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Cell organelles and debris were sedimented by sequential centrifugation: 600 × g for 20 min followed by 30,000*g* for 60 min. The resultant supernatant fluid was labeled 30-s. This preparation then was centrifuged at 100,000*g* for 2 hr at 4° and designated 100-s. The 100-s preparation was dialyzed against 100 vol of PBS at 4° over a 24-hr period. PBS was changed four times at 6-hr intervals. The product was designated 100-s,d. All preparations were passed through a 0.45 μm filter (Millipore Corporation, Bedford, MA) and stored at −60°.

3 M KCl extraction. The methods were adapted from those described by Metzler *et al.* (9). Seven milliliter of a solution containing 4.27 *M* potassium chloride and 0.005 *M* potassium phosphate buffer, pH 7.4 was added to 3 ml (4°) of cell suspension. After incubation for 18 hr the solution was clarified by centrifugation at 30,000*g* for 1 hr. The pellet was discarded and the supernatant fluid was dialyzed against two changes of 100 vol of PBS at 4°. The slight precipitate that formed was removed by centrifugation at 30,000*g* for 60 min. The product was designated 30-k. Further centrifugation, filtration, and dialysis were the same as described above thus yielding preparations designated 100-k and 100-k,d, respectively.

Sephadex filtration. Solubilized antigen preparations were fractionated by column chromatography employing either Sephadex G-150 or Sephadex G-200 (Pharmacia Fine Chemicals; Uppsala, Sweden). Samples were fractionated on 0.9 × 30 cm columns. One-half milliliter fractions were collected every 30 min. Columns were eluted with 0.05 *M* sodium phosphate buffer with 0.10 *M* sodium chloride, pH 7.4 at 4°. Protein concentrations were determined by the method of Lowry *et al.* (10). All columns were calibrated with blue dextran (mol wt = 10^6), bovine serum albumin (mol wt = 69,000), and egg white lysozyme (mol wt = 13,800).

Electron microscopy. At various stages of antigen preparation samples were examined by the negative staining technique. Droplets of solubilized preparations were deposited on formvar carbon coated, two-hundred mesh, grids. After blotting specimens dry they were negatively stained with 2% phosphotungstic acid (aqueous) for 30 sec. Samples were examined in a JEOL-100B electron microscope (Medford, MA) operating at 60 KV.

Results. Solubilization of antigens. Antigens were solubilized from I_b cells by either low frequency sonication (5 min, 10 KHz, 1.25 ma, 4°) or 3 *M* KCl extraction (18 hr, 4°) as described. Decreases in protein concentration during fractionation were compared using the starting preparations 30-s and 30-k as the reference. With the sonicates there was approximately a 12% decrease in protein between the 30-s and 100-s preparation with an additional 17% decrease after dialysis. Overall, there was approximately a 27% decrease between the 30-s and 100-s,d products. For the KCl extracts the decrease in protein concentration between the 30-k and 100-k preparation was approximately 7% followed by an additional 28% decrease after dialysis. Overall, there was a 29% decrease in protein content between the 30-k and 100-k,d products. Electron microscopic examination of the various preparations showed that the 30-s preparation contained numerous cell membrane fragments; C-type particles were rare. Corresponding 30-k preparations did not contain detectable C-type virus particles and membrane fragments were rare. The 100-s preparations did not contain detectable C-type virus particles; membrane fragments were rare. The 100-k preparations did not contain detectable membrane fragments or C-type virus particles.

Sephadex G-150 gel filtration. These experiments were done to determine whether solubilized preparations were stable, recoverable from gels, and to indicate the major molecular species that were immunogenic or paralytogenic. The 30-s preparation containing three major molecular species (Fig. 1A). Peak 1 (tubes 15–21) consisted mainly of the void volume, mol wt ≥ 150,000. Peaks 2 (tubes 22–33) and 3 (tubes 34–39) had mol wt of approximately 69,000 and 14,000, respectively. The 30-k preparation contained only 2 major molecular species corresponding to mol wt of approximately 60,000 (tubes 25–33) and ≤14,000 (tubes 34–40), respectively. The slight shoulder (tube 21) in the first peak suggested the occurrence of a third unre-

solved molecular species. When the 30-s preparation was ultracentrifuged at 100,000*g* for 2 hr (Fig. 1B), the profile for the 100-s preparation was essentially the same as in Fig. 1A except for lesser amounts. However, four peaks were found in the 100-k preparation. Figure 1C shows that dialysis removed the lower molecular weight species from both the 100-s and 100-k preprations.

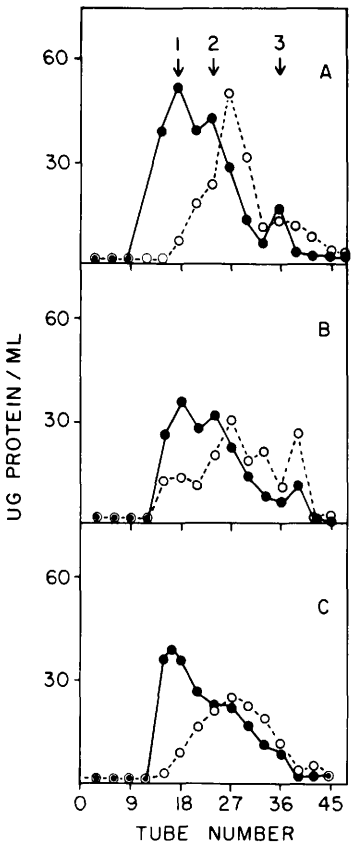

FIG. 1. Sephadex-150 gel filtration of solubilized preparations. Columns (0.9 × 30 cm) were equilibrated with 0.05 *M* sodium phosphate buffer containing 0.10 *M* sodium chloride, pH 7.4. Columns were calibrated (arrows, left to right) with the dextran (mol wt, 2 × 10^6), bovine serum albumin (mol wt, 69,000) and egg white lysozyme (mol wt, 14,000). Each column received 0.3 ml of preparation (0.725 mg protein/ml); 0.5 ml fractions were collected every 30 min (4°) by elution with the described buffer. The Lowry method (10) was used for protein determinations. (A) shows 30-s (●——●) and 30-k (○- - -○); (B) 100-s (●——●) and 100-k (○- - -○); and (C) 100-s,d (●——●) and 100-k,d (○- - -○).

Induction of IPE by solubilized preparations. Table I shows that the 30-s and 30-k preparations were fully paralytogenic at ≥1 μg. The 100-s, 100-k, 100-s,d, and 100-k,d paralyzed about 50% of mice at the 100 μg level but not at the 1 μg concentration. Control preprations from normal C58 mouse spleens, or from spleens of mice with spontaneous leukemia, were not paralytogenic. Table II shows that preparations eluted from Sephadex G-150 columns elicited IPE.

Immunization of mice to IPE. Three-month-old normal C58 mice received from 1 to 4 weekly injections ip of solubilized preprations. Four weeks later mice were tested for their capacity to resist the induction of IPE when challenged by the injection of x-irradiated I_b cells. Control mice received either HBSS or normal syngeneic spleen cells in place of the immunizing preparation. Table III shows that all test preparations were immunogenic.

TABLE I. INDUCTION OF IPE BY SOLUBILIZED LINE I_b CELL PREPARATIONS.

Cell preparation	Protein (μg) injected ip	Fraction of mice that[a] developed IPE
30-s	100	5/5
	10	8/8
	1	5/7
30-k	100	7/9
	10	7/9
	1	8/8
100-s	250	15/17
	100	3/8
	1	1/9
100-k	250	4/4
	100	5/7
	1	0/7
100-s,d	250	9/9
	100	6/10
	1	0/10
100-k,d	250	4/4
	100	6/9
	1	0/10
NMS-30-s[b]	250	0/19
SL-30-s[c]	500	0/5

[a] Number of mice with IPE/number injected. Five-month-old indicator mice received 525R 1 day before ip injection of 1 ml of each preparation. Mice were scored for paralysis over a 30-day period.

[b] Normal mouse spleen cells were used in place of I_b cells.

[c] Spleen cells were obtained from C58 mice (6–12 months old) with spontaneous leukemia.

TABLE II. INDUCTION OF IPE BY 30-S AND 30-K FRACTIONS ELUTED FROM SEPHADEX G-150 COLUMNS.

Eluate[a]	μg or ng of protein injected ip	Fraction paralyzed[b]	
		30-s	30-k
Peak 1	25 μg	16/16	Not done
(mol wt ≥ 150,000)	2.5 μg	14/15	
	250 ng	10/15	
	25 ng	4/16	
	2.5 ng	0/14	
Peak 2	25 μg	10/20	12/21
(mol wt ≈ 69,000)	2.5 μg	0/13	3/22
	250 ng	1/14	0/16
	25 ng	0/12	0/17
	2.5 ng	0/17	0/17
Peak 3	25 μg	9/18	Not done
(mol wt ≈ 14,000)	2.5 μg	0/16	
	250 ng	0/16	
	25 ng	0/14	
	2.5 ng	0/13	
30-s (unfractionated)	250 μg	23/25	
HBSS		0/22	

[a] Eluates from five tubes of the central portion of each peak were combined.

[b] See footnote a, Table I.

TABLE III. IMMUNIZATION OF C58 MICE TO IPE BY SOLUBILIZED I_b CELL PREPARATIONS.

Solubilized cell preparation	Number of weekly[a] i.p. injections	Fraction of mice that developed IPE[b]
30-s	4	0/10
	3	0/10
	2	3/8
	1	2/10
30-k	4	1/9
	3	0/7
	2	0/5
	1	1/9
100-s[c]	1	0/10
100-k	1	0/10
NSC[d]	1	12/20
HBSS	1	13/24

[a] Each 1 ml injection contained 1 mg of protein.

[b] See footnote a, Table I. Data were compiled from two replicate experiments.

[c] Mice in the 100-s or 100-k groups received a single injection 2 weeks before challenge.

[d] x-Irradiated normal spleen cells were used for immunization.

Immunization to transplantable leukemia. C58 can be immunized to purified I_b cell membranes (11) providing that optimum dose regimens are used. Immunization was most effective when 10^3 viable I_b cells were injected with purified membrane fractions. A similar approach was used to determine the immunogenicity of the solubilized line I_b cell antigens. Table IV shows that 1–10 mg of 30-s or 1–5 mg of 30-k were immunogenic when injected with either 10^2 or 10^3 viable I_b cells. When mice received 2–3 ip injections of 30-s alone, spaced 4 days apart, and were challenged with 10^3 viable I_b cells 15 days later, 8/20 survived challenge. However, neither the 100-s or 100-k preprations were immunogenic as tested (Table IV).

Discussion. These experiments compared the effectiveness of solubilizing antigens from line I_b cells by low energy sonication and 3 *M* KCl extraction. The immediate objective was to determine which technique was superior and whether the products obtained elicited IPE or were immunogenic. The more long term objective is to determine whether the same molecular species of antigen that elicit IPE also are immunogenic to IPE and transplanted line I_b leukemia. In assessing results it is relevant to consider an appropriate definition of the term "soluble." Reisfeld and Kahan (8) suggested that solubility be defined to mean that preprations are free of membrane fragments as determined by electron microscopy, that they penetrate into chromatographic gels, and that they do not lose antigenic activity after "extensive ultracentrifugation at 130,000*g*." We found membrane fragments, and the C-type virus par-

TABLE IV. IMMUNIZATION OF C58 MICE TO TRANSPLANTABLE LEUKEMIA.

Solubilized preparation	Admixture[a]		Survivors[b]
	Mg solubilized antigen	Viable I_b cells	
30-s	10	10^3	13/16
30-s	5	10^3	9/10
30-s	5	10^2	9/10
30-s	1	10^2	8/10
30-k	5	10^3	3/3
30-k	1	10^2	3/15
100-s	5	10^3	0/16
100-k	5	10^3	0/7

[a] Mice that received a single ip injection of each admixture were challenged with either 10^2 or 10^3 LD100 of viable I_b cells.

[b] Number of mice that survived immunization and challenge/number tested. When control mice received 10^7 viable normal C58 spleen cells in place of the soluble antigen none survived the immunization.

ticles associated with line I_b cells, to be useful markers to monitor purification by electron microscopy. Interestingly, the 3 *M* KCl preparations were essentially free of membrane fragments after centrifugation at 30,000*g* for 1 hr and C-type particles were not detected. Sonicates were not free of membrane fragments even after centrifugation at 100,000*g* for 2 hr. Metzgar *et al.* (12) have shown that centrifugation at 100,000*g* for 4–6 hr sediments membrane vesicles in KCl extracts of human leukemic cells. Such results suggest that it is difficult to free solubilized preparations from membrane fragments. The current results suggest that 3 *M* KCl extraction is a superior approach in this regard.

Within the context of the current studies the Sephadex gel filtration experiments were not carried out in sufficient detail to permit firm conclusions to be drawn concerning the precise molecular species possessing biological activity. It is clear, however, that the procedures used for purification did not destroy the biological activity of the various products. The Sephadex G-150 gel filtration data indicate that the high molecular weight "excluded volume" seen with sonicated preparations was greatly diminished in the 3 *M* KCl extracts. Several lines of evidence suggest that paralytogenic activity was not exclusively associated with the void volume. Table II presents data indicating that IPE was elicited by molecular species found in peak 2 (mol wt $\approx$ 69,000) and 3 (mol wt $\approx$14,000). These results confirm our earlier work (13) which showed that serum, obtained from mice 1 day after the ip injection of 10^7 inactivated I_b cells, contained molecular species of a size range of 64,000–165,000 that elicited IPE. Considered together the results indicate that molecular species with a size range less than 150,000 daltons may elicit the disease. From the current studies it appears that by the use of 3 *M* KCl extracts and preparative gel electrophoresis it should be possible to identify definitively the molecular species responsible for eliciting IPE. In this regard one key advantage of the IPE model is that the exquisite sensitivity of the assay for the disease makes it easy to trace and quantify biological activity during various steps of purification.

Since sensitive methods were worked (1–3) out for immunizing C58 mice to line I_b leukemia and IPE it was possible to test whether immunogenicity was lost during solubilization and purification of antigens. Table III shows that a single ip injection of 1 mg of the 30-s, 30-k, 100-s or 100-k preprations immunized mice to IPE. Table IV shows that the 30-s and 30-k preparations also immunized mice to transplantable leukemia but that the 100-s and 100-k preparations were not immunogenic under the simple test conditions that were used. Considered together, however, the results presented in Tables III and IV provide a basis for determining whether the same molecular species can be both immunogenic and paralytogenic in the line I_b-C58 model. The crucial difference in a protective response, compared with a paralytogenic one in the IPE model, appears to be governed (6) by a loss in a 350R resistant T cell subpopulation (presumably a suppressor T cell) which is lost spontaneously as C58 mice age.

Summary. The relative effectiveness of sonication and 3 *M* KCl extraction for solubilizing antigens from line I_b malignant lymphocytes were compared. Electron microscopic studies provided evidence that 3 *M* KCl extracts contained fewer membrane fragments and more closely approximated a rigorous definition of "soluble." Sephadex G-150 gel filtration data indicated that the solubilized preprations contained three main peaks, viz, the void volume, a molecular species $\leq$69,000 and a molecular species $\leq$14,000. Fractions eluted from Sephadex columns elicited immune polioencephalomyelitis in indicator mice. Solubilized preparations immunized mice to the disease. Preparations containing membrane fragments immunized C58 mice to transplantable line I_b leukemia.

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