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The Use of Subcellular Fractions to Raise Anti-Lymphocytic Serum

E. M. LANCE, P. J. FORD AND M. RUSZKIEWICZ

National Institute for Medical Research, Mill Hill, London, N.W.7

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Summary. Subcellular preparations of mouse thymocytes were compared with respect to their power to raise in rabbits antisera which prolonged the survival of skin homografts. Of the fractions studied thymocyte 'membrane' gave the most consistently good results, whereas thymocyte 'mitochondria' gave rise to sera which were toxic in high dose. Whole mouse liver homogenate or liver subcellular fractions did not elicit potent antisera. It is concluded that thymocyte 'membrane' best fulfils the requirements for an ideal material for immunization thus far studied and provides a convenient starting point for further purification.

INTRODUCTION

Subcellular fractions were used to raise 'anti-lymphocytic' serum (ALS) to see whether the pertinent antigens reside largely or wholly in a discrete anatomical compartment, and secondly to provide better antigenic preparations for immunization. At present most methods rely on the use of whole cell inocula which, regardless of source, give rise to a miscellany of antibodies some unwanted and others thoroughly undesirable (e.g. those directed against erythrocytes). An approach to this problem has been the use of extensive absorption procedures which are time-consuming, expensive and may not be entirely effective. A better answer would be the use of preparations for immunization which already had been divested of the bulk of contaminating antigens. Furthermore any subcellular fraction proved rich in pertinent antigens might well serve as the starting material in the search to isolate and identify the target of anti-lymphocyte antibodies.

MATERIALS AND METHODS

Preparation of subcellular fractions

Experiment 1. A single-cell suspension was prepared from the thymuses of 300 CBA weanling mice (wet weight 17·7 g; total cell yield 2×10^{11}), washed twice in medium A (0·15 M NaCl, 5 mM Tris, pH 7·4) and once in medium B (0·12 M KCl, 5 mM Tris, pH 7·4, 1 mM MgCl), and finally suspended in 9 volumes of medium B. The cells were disrupted by intracellular cavitation of nitrogen by exposing them to a pressure of 1000 lb/in^2 for 20 minutes with mechanical stirring. The recovered homogenate, containing 90–95 per cent totally disrupted cells, as observed by phase contrast microscopy, was made 1 mM with

respect to EDTA, pH 8·6. Four fractions were prepared from this homogenate by differential centrifugation. Centrifugation at 2500 *g* average for 15 minutes produced a pellet (fraction D). The supernatant was further centrifuged at 10,000 *g* average for 15 minutes to yield a pellet (fraction C) and a supernatant which was finally centrifuged for 1 hour at 100,000 *g* average to give a pellet (fraction B) and a supernatant (fraction A). The pellets were re-suspended in 8 ml of medium C (0·25 M sucrose, 1 mM Tris, pH 8·6) and stored at −20°.

The fractions resulting from this procedure were identified through analysis for enzyme markers and by observation under phase contrast microscopy (see Experiment 2). While these fractions were by no means entirely free of cross contamination they were felt to consist largely of: B, microsomal and plasma membrane fragments; C mitochondria; D, whole or disrupted nuclei and a small percentage (5 per cent) of undisrupted cells (Table 1).

TABLE 1

ENZYME CONTENT OF THYMUS SUBCELLULAR FRACTIONS—EXPERIMENT 1

Fraction	Total protein (mg)	S.A.* 5′-Nucleotidase	Esterase	Succinic dehydrogenase	Acid phosphatase
A. Supernatant	285	42·2	4·48	1·64	6·52
B. 'Microsome-membrane'	53	64·5	6·96	1·09	19·10
C. 'Mitochondrial'	60	58·5	2·28	4·62	6·99
D. 'Nuclear'	40	5·4	0·15	0·53	0·36

* Specific activity: 5′-nucleotidase as μM Pi/min/mg protein; esterase as OD 420 mμ/min/mg protein; succinic dehydrogenase as OD 600 mμ/min/mg protein; acid phosphatase as OD 420 mμ/min/mg protein.

Experiment 2. A single-cell suspension was prepared from the thymuses of 300 CBA weanling mice (wet weight 18·8 g; cell yield $5{\cdot}6 \times 10^{10}$) washed twice in medium A and once in medium B and resuspended in 9 volumes of medium B. The cells were disrupted as above and the recovered homogenate made 1 mM with respect to EDTA, pH 8·6, and 8 per cent with respect to sucrose. After differential centrifugation as above, fractions B and C were resuspended in 6 ml of medium B by gentle agitation in a loose fitting Dounce homogenizer. Two-millilitre aliquots of these fractions were layered over linear sucrose gradients (30 ml of 15–65 per cent sucrose in 0·2 M KCl, 50 mM Tris, pH 7·4) and centrifuged at 25,000 rev/min (67,000 *g* average) at 0° in a Spinco SW 25 rotor for 15 hours. The gradients were fractionated to give approximately thirty 1-ml fractions which were characterised by assaying for protein (Lowry, Roseborough, Farr and Randall, 1951), 5′-nucleotidase (Michell and Hawthorne, 1965), succinic dehydrogenase (Earl and Korner, 1965), acid phosphatase (Schmidt, 1955), esterase (Earl and Korner, 1965) and DPNH diaphorase (Wallach and Kamat, 1966) (Table 2, Fig. 1). Sucrose concentration was estimated by refractometry.

The tubes containing the peak enzyme activities of 5′-nucleotidase (cell membrane fraction), DPNH diaphorase and esterase (microsome fraction) and SDH (mitochondrial fraction) were pooled from the duplicate gradients and the material recovered by centrifugation. Representative samples of each of these fractions were kindly examined by Dr

J. A. Armstrong using electron microscopy (Fig. 2) showing them to be extremely homogeneous preparations of (a) smooth membranes, (b) smooth membranes plus some rough membranes, and (c) mitochondria, diagnoses consistent with their known enzyme content.

TABLE 2

ENZYME CONTENT OF SUBCELLULAR FRACTIONS FROM THYMUS CELLS–EXPERIMENT 2

Fraction	Total protein (mg)	Specific activity				
		5′-Nucleotidase	Esterase	βDPNH diaphorase	SDH	Acid phosphatase
Membrane	10·0	98	0·18	0·25	0·27	1·39
Microsome	9·8	47	0·26	0·61	0·69	1·22
Mitochondrial	17·0	36	0·56	0·70	1·19	1·18

Results are expressed in the same form as in Table 1. βDPNH diaphorase as OD 340 mμ/min/mg protein.

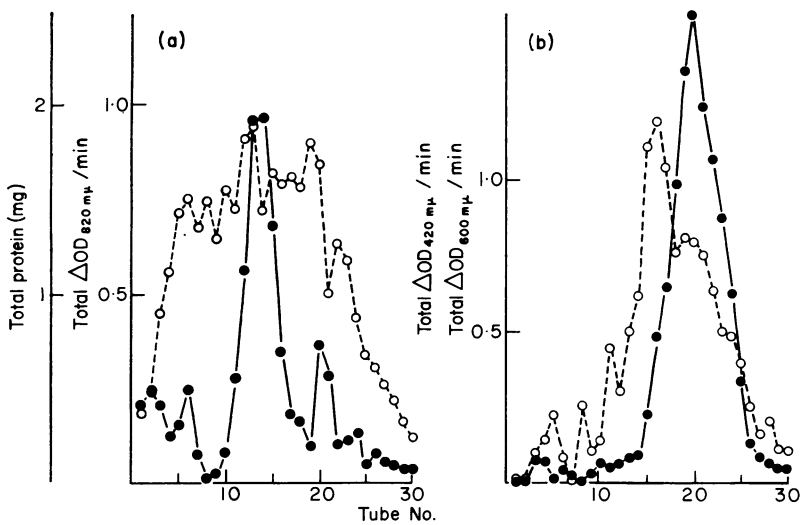

FIG. 1. The distribution of protein and enzyme activity in sucrose gradients of thymus subcellular fractions (Experiment 2). Assays were performed on the fractions from each gradient independently; however the results are portrayed as summed for clarity of presentation. (a) Total protein (○) and the enzyme 5′-nucleotidase (●, membrane marker) activity. (b) Esterase activity (microsomal, ○) and succinic dehydrogenase activity (mitochondrial marker, ●). Tubes number 12–14 were pooled as 'membrane' fraction, 15–17 as 'microsome' fraction, and 18–23 as the 'mitochondrial' fraction (see text for details of preparation).

Experiment 3. Liver tissue (20 g) from twelve CBA mice was homogenized with ten strokes of a loose-fitting Dounce homogenizer in 50 ml of 1 mM $NaHCO_3$, pH 8·6 (Neville, 1960), and layered over a sucrose gradient (200 ml, 5 per cent; 200 ml, 30 per cent; 750 ml, linear with volume 30–70 per cent sucrose in 1 mM $NaHCO_3$) in a MSE A type zonal rotor running at 500 rev/min at 0° in a MSE 6L centrifuge. An overlay of 70 ml was pumped in and the rotor speed increased to 5000 rev/min for 2 hours resolving the sample into five distinct bands observable by scattered light. The rotor was slowed to 500 rev/min

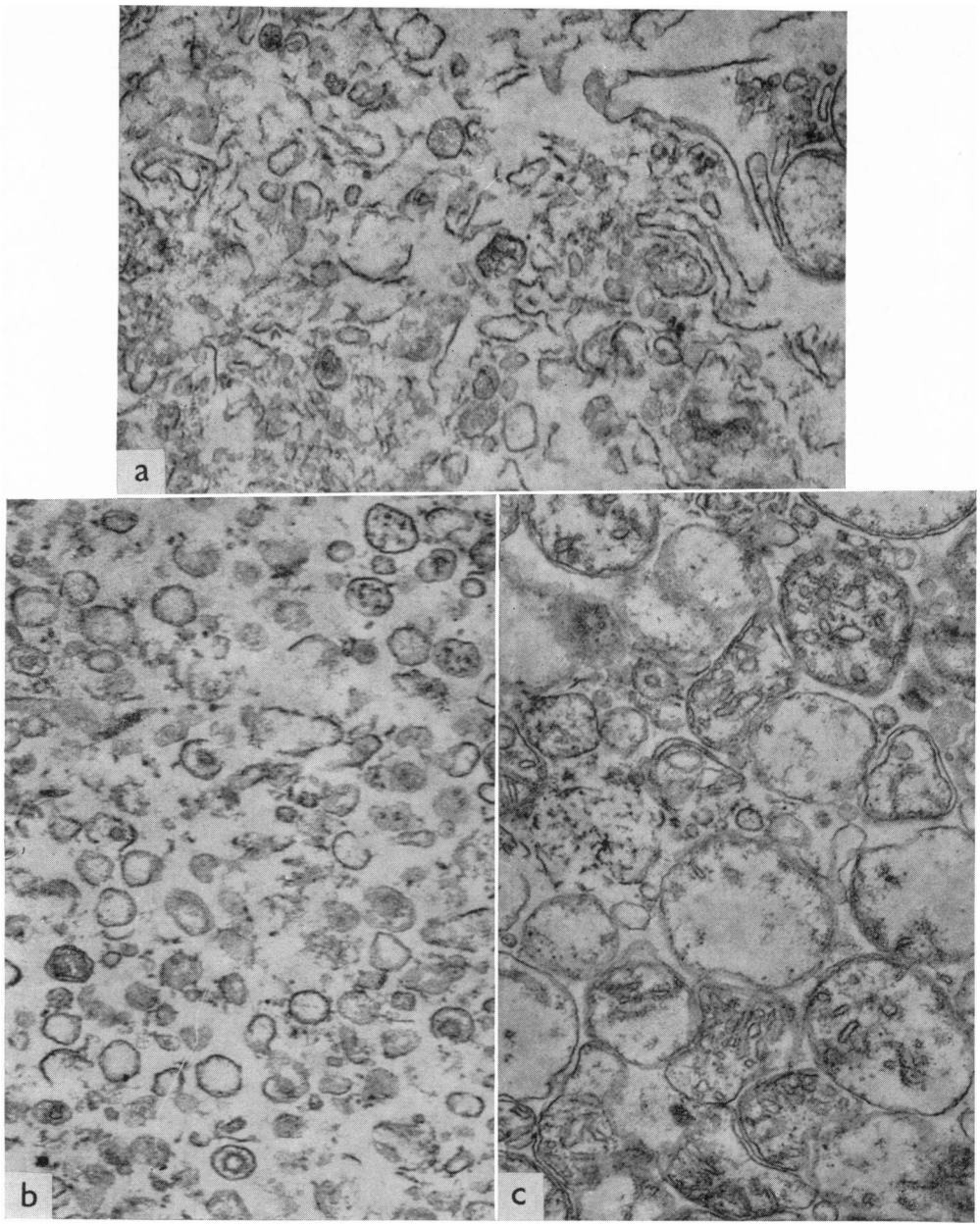

FIG. 2. Electron micrographs of the fractions produced in Experiment 2. (a) 'Membrane' fraction (× 38,400) consisting mainly of sheets of membranous material with very few if any ribosome particles and a few vesicles. (b) 'Microsome' fraction (× 38,400) consisting predominantly of vesicular membranous material and some ribosomes. (c) 'mitochondrial' fraction containing swollen mitochondria with characteristic cristae and double external membrane, plus some small vesicular material. The main difference between the membrane and microsome fractions is the character of the membranous material. In the membrane fractions the material has the form of open sheets whereas in the microsome fraction the material is mainly vesicular.

and the gradient pumped out and collected in 10 ml fractions. The three fractions representing the peaks of each of the five observable bands were pooled and the particulate matter recovered by centrifugation at 40,000 *g* average for 2 hours, resuspended in 8 ml of 1 mM $NaHCO_3$ and stored at −20°. Each fraction was characterized by electron microscopy (kindly performed by Dr J. A. Armstrong) and by assaying protein RNA (Fleck and Munro, 1962), DNA (Burton, 1956), phospholipid phosphorus (Ames, 1966; Tata, 1967), succinic dehydrogenase (Pennington, 1961) and esterase (Nachlas and Seligman, 1949) (Table 3). The fractions identified in this way and later used were: (1) whole liver

TABLE 3

ENZYME CONTENT OF LIVER CELL FRACTIONS

Fraction	Protein*	RNA*	DNA*	Phospholipid phosphorus*	Succinic† dehydrogenase	Esterase‡	Volume (ml)
1. Whole homogenate	50	1·10	0·210	0·165	0·69	0·04	70
2. Supernatant	12·25	0·54	0·115	0·112	0·00	0·25	8
3. Small microsomes	7·00	0·55	0·066	0·062	0·39	0·28	8
4. Large microsomes	5·75	0·43	0·10	0·054	2·17	0·64	8
5. Mitochondria	6·75	0·32	0·008	0·142	9·80	0·37	8
6. Plasma membrane	2·75	0·11	0·006	0·118	1·23	1·09	8
7. Whole cells and nuclei	2·75	0·17	0·950	0·144	1·64	1·60	8

* Protein, RNA, DNA and phospholipid phosphorus as mg/ml of sample.
† SDH activity as OD 490 mμ/min/mg protein.
‡ Esterase activity as OD 540 mμ/min/mg protein.

TABLE 4

IMMUNIZATION SCHEDULE (EACH RECIPIENT GROUP CONSISTED OF TWO RABBITS)

Material injected	Day 0 first injection	Day 14 second injection	Day 21	Day 28 third injection	Day 35	Day 42 fourth injection	Day 49
Experiment 1: Thymocyte fractions			↑		↑		↑
A. Supernatant	5·8*	5·8	|	2·9	|	2·9	|
B. Microsome membrane	5·0	5·0	|	2·5	|	2·5	|
C. Mitochondrial	6·0	6·0	|	3·0	|	3·0	|
D. Nuclear	4·0	4·0	|	2·0	|	2·0	|
Experiment 2			|		|		|
A. Membrane	1·8	1·8	First	0·9	Second	0·9	Third
B. Microsome	1·8	1·8	bleed	0·9	bleed	0·9	bleed
C. Mitochondrial	1·8	1·8	|	0·9	|	0·9	|
Experiment 3: Liver fractions			|		|		|
A. Whole liver homogenate	2·0	2·0	|	—	|	—	|
B. Supernatant	12·5	12·5	|	—	|	—	|
C. Microsomes	7·0	7·0	|	—	|	—	|
D. Mitochondria	6·8	6·8	|	6·8	|	—	|
E. Membranes	2·8	2·8	|	2·8	|	—	|
F. Nuclei	2·8	2·8	↓	2·8	↓	—	↓

* All injections were by the intravenous route and the dosage is given as mg protein content.

cell homogenate, (2) supernatant, (3) small microsomes, (4) large microsomes, (5) mitochondria, (6) membranes, and (7) nuclei.

Immunization for production of antisera

New Zealand white rabbits were immunized by the intravenous injection of cell fractions according to the schedule outlined in Table 4. Sample bleeds during the course of

immunization were obtained from the ear vein but did not exceed 15 ml from any one animal. After the last immunization rabbits were exsanguinated by cardiac puncture. The sera for each group were pooled and inactivated by heating to 56° for 30 minutes, sterilized by Seitz filtration and stored at −20°. Just prior to use, the sera were thawed rapidly.

Assay for potency

Sera were assayed for potency *in vivo* by the ability to prolong the life of skin homografts according to the method of Levey and Medawar (1966). CBA male mice received A strain tail skin grafts followed by the subcutaneous injection on the 2nd and 5th post-graft days of 0·5 ml of the respective serum under assay. Bandages were removed on the 10th post-graft day and life tables constructed for the homograft survival. The results are expressed as the mean expectation of life (MEL) for the various groups. In our laboratories the MEL for first set skin grafts across this strain combination has been established over the course of many experiments as 11·6±1·3 days.

The *in vitro* potency of these sera was assayed by an adaptation of the cytotoxicity test described by Wigzell (1965). Single-cell suspensions were prepared from the lymph nodes of CBA male mice in Hanks's balanced salt solution with 0·1 per cent bovine serum albumin (BSA) added. The cells were washed three times and the final suspension was adjusted to 1×10^8 cells/ml. $Na_2Cr^{51}O_4$ (20–30 μc/ml) was added and the cells were then incubated at 37° for 30 minutes in an atmosphere of 5 per cent CO_2 and 95 per cent air. After incubation the cells were washed twice in cold medium and kept at 4° for 30 minutes. The final resuspension was in veronal buffered saline with 0·1 per cent BSA to a concentration of 5×10^6 lymphocytes/ml. For the cytotoxic titration, 0·1 ml of the ^{51}Cr-labelled lymphocytes was added to an equal volume of serially diluted antiserum followed after 5–10 minutes by the addition of 0·1 ml of adsorbed and suitably diluted rabbit complement, and the mixture was incubated at 30° for 30 minutes. The reaction was stopped by the addition of 1·1 ml of cold saline and the tubes were centrifuged at 4°. One-millilitre aliquots of the supernatant were removed and the radioactivity determined in a Packard auto-gamma counter. Titres are expressed as that dilution of antiserum causing half maximal liberation of ^{51}Cr from the labelled lymphocytes (50 per cent lysis end point).

RESULTS

The results of the first experiment are summarized in Table 5. Immunization with the

TABLE 5

MEAN SURVIVAL OF GRAFTS IN MICE TREATED WITH ALS PREPARED AGAINST FRACTIONS OBTAINED IN EXPERIMENT 1

Thymocyte fraction	First bleed	Second bleed	Third bleed
A. Supernatant	13·4±1·0* (160)†	13·8±1·8 (100)	13·7±1·2 (640)
B. Membrane-microsome	17·5±2·6 (2200)	24·0±4·6 (2000)	21·1±3·5 (1600)
C. Mitochondria	20·3±3·8 (6400)	23·3±3·6 (2000)	22·8±3·7 (1600)
D. Nuclear	21·3±3·7 (5120)	18·7±1·9 (500)	13·8±2·8 (400)

* MEL (mean expectation of life) based in all assays on groups of seven to ten CBA male mice bearing A strain tail skin grafts.

† Cytotoxic titre expressed as the reciprocal of that dilution which causes half maximal liberation of ^{51}Cr from the labelled lymphocytes.

supernatant fraction gave sera which had minimal activity at all test intervals with regard to both skin graft survival and cytotoxic titre. The 'nuclear' fraction produced a moderately potent ALS at the first bleed which was correlated with a high cytotoxic titre. However, with repeated immunization there was a falling off in activity of both these parameters which at the end of the experiment was minimal. On the other hand both the 'membrane–microsome' and the 'mitochondrial' fractions yielded potent antisera which did not change significantly in strength during repeated immunization. The cytotoxic titres for these sera were generally high.

It should be emphasized that all fractions were capable of eliciting antibodies which were cytotoxic to mouse lymphocytes and which prolonged skin graft survival. Repeated immunization had a variable effect depending upon the fraction used. Finally, although within a group the cytotoxic titres correlated roughly with the *in vivo* potency, the former could not be used to predict the value of the latter in a general way. This point is illustrated by a comparison of the values for the first bleed of fraction B with the last bleed of fraction C or indeed the second and third bleeds of fraction C with each other.

TABLE 6

MEAN SURVIVAL OF GRAFTS IN MICE TREATED WITH ALS PREPARED AGAINST FRACTIONS OBTAINED IN EXPERIMENT 2

Thymocyte fraction	First bleed	Second bleed	Third bleed
Membrane	22·5±3·7* (6400)†	28·1±4·6 (3800)	23·7±4·3 (3840)
Microsome	19·6±3·3 (3200)	14·6±2·0 (2560)	22·5±5·1 (1600)
Mitochondria	19·8±4·6 (3200)	17·0±4·2 (2560)	16·8±2·0 (2140)

* MEL (mean expectation of life) based in all assays on groups of seven to ten CBA male mice bearing A strain tail skin grafts.

† Cytotoxic titre expressed as the reciprocal of that dilution which causes half maximal liberation of ^{51}Cr from the labelled lymphocytes.

In the first experiment rabbits were immunized with aliquots of the various fractions intended to correspond roughly with the yield of 1×10^9 thymocytes (the number of cells used to raise routine ALS in our laboratory). In the second experiment attention was focused on three thymocyte fractions which had been highly purified: 'membrane', 'microsome', and 'mitochondrial'. An additional refinement was the immunization with dosages standardized with respect to protein content. The overall results are set forth in Table 6. All three fractions gave sera at all periods active both *in vivo* and *in vitro*. Sera obtained by immunization with the membrane fraction gave the most consistently good results while that elicited by the mitochondrial fraction tended to deteriorate with repeated immunization.

In the third experiment sera raised by the injection of whole liver homogenate and liver subcellular fractions were tested. No sera were obtained which were demonstrably effective *in vivo* and the highest *in vitro* titre recorded was 1:160.

During the course of these experiments we were somewhat surprised to observe that none of the animals was made clinically ill by the administration of sera gathered after the fourth immunization. It has been our general experience that sera raised according to this schedule with whole thymocytes invariably require absorption with erythrocytes prior to use. Therefore we performed a toxicity test comparing the third bleed sera from Experiment 2 with serum raised against whole thymocytes corresponding to the third

bleed (hyperimmune serum). The scheme of this test and the results are given in Table 7. It is apparent that hyperimmune serum raised against whole thymocytes is uniformly and rapidly lethal in both 1- or 2-ml quantities. Hyperimmune serum raised against thymocyte mitochondria are toxic at both dosage levels. Gross haemoglobinuria was evident within 12 hours of administration and two-thirds of the animals succumbed within 48 hours. On the other hand sera raised against the membrane or microsomal fractions were entirely free of toxicity. Since the lethal effects of the serum produced by repeated immunization with mitochondria seemed to be due to the presence of antibodies directed against erythrocytes,

TABLE 7

RESULTS OF *in vivo* TOXICITY TEST: COMPARISON OF SERA RAISED WITH WHOLE CELLS *versus* THOSE RAISED WITH SUBCELLULAR FRACTIONS

Antigen source	Dose* (ml)	Day 1	Day 2	Day 3	Day 4	Day 5
Whole thymocytes hyperimmune	1	0/3†				
	2	0/3				
Thymocyte membrane hyperimmune	1	3/3	3/3	3/3	3/3	3/3
	2	3/3	3/3	3/3	3/3	3/3
Thymocyte microsome hyperimmune	1	3/3	3/3	3/3	3/3	3/3
	2	3/3	3/3	3/3	3/3	3/3
Thymocyte mitochondria hyperimmune	1	3/3 HgB Sick	3/3 HgB Sick	3/3 Sick	3/3 Sick	3/3 Sick
	2	3/3 HgB Sick	1/3 HgB Sick	1/3 Sick	1/3 Sick	1/3 Sick

Thymocyte subcellular fractions used to raise the sera described in Experiment 2 and the sera tested here are those obtained at the third bleed.

The serum raised with whole thymocytes was prepared to correspond with immunization schedules and dosages represented by the third bleed (hyperimmune) in Experiment 2. This serum more than doubled the survival of A strain skin on CBA mice after absorption with erythrocytes.

* All sera administered intraperitoneally on day 0 of study. HgB = Gross haemoglobinuria.

† The fraction of animals surviving on any given day.

we tested the haemagglutination titre of these sera comparing them with a routine ALS (two-pulse) raised by the administration of whole cells on two occasions. Routine (two-pulse) ALS is ordinarily non-toxic when tested as above and does not cause haemoglobinuria. The titres found for routine ALS and hyperimmune sera raised with thymocyte 'membranes' or 'microsomes' were identical (1:80). The titre of haemagglutinins in the hyperimmune serum raised against 'mitochondria' was considerably higher (1:1280).

DISCUSSION

The results of this study strongly suggest that the antigens capable of eliciting a potent ALS do not reside exclusively in any one anatomical subfraction. Although the range of potency varies considerably, all fractions prepared from thymocytes could be used to

raise active antisera. This observation is in accord with those of Levey and Medawar (1966) who fractionated thymocytes by chemical means into 'crude soluble', 'crude insoluble', and various 'nucleoprotein' fractions. All were capable of raising potent antisera; however, the fraction containing membrane antigens (crude insoluble) was the most effective.

These findings could be explained if the fractions were impure to any great extent. While we believe that minor contamination did occur, the electron microscopic and enzyme analyses provide good evidence of homogeneity especially in Experiment 2. An alternate possibility is that the relevant antigens are in fact found throughout the various cell compartments but the quantitative distribution varies from site to site.

Nonetheless, it seems clear that the 'membrane' fraction provides a consistently good source of these antigens and can be used to produce an antiserum which is non-toxic and whose potency does not decay after repeated immunization. The phenomenon of decline in activity following repeated immunization was first reported by Levey and Medawar (1966). It is again evident in the present study (fraction D in Experiment 1, and the 'mitochondrial' fraction in Experiment 2). One reasonable interpretation of this observation is that whole cells and certain subcellular preparations contain antigens irrelevant to the action of ALS but which tend to preoccupy progressively the attention of the immunized animal. Removal of such material might account for the absence of decay in, for instance, animals immunized with 'membrane' alone.

The 'mitochondrial' fraction gave rise to high titres of antibodies directed against erythrocytes and proved lethal upon intraperitoneal injection into mice. A likely explanation for this finding is that red cell ghosts tend to migrate on a gradient with mitochondria although such ghosts were not seen on electron microscopy. An alternative explanation is that erythrocytes and mitochondria share common antigens. Regardless of reason, this finding renders this fraction less suitable for use in the production of ALS and its removal from the immunizing material seems worthwhile.

Although it is generally accepted that ALS is directed against lymphocyte antigens, Levey and Medawar (1966) have reported the use of other tissue sources to raise active antisera, suggesting that the relevant antigens are not the exclusive property of lymphoid tissue. In this regard it was somewhat disappointing that neither liver cells nor liver subcellular fractions were capable of eliciting potent antisera.

An ideal material for ALS manufacture should have at least three properties. It must be rich in the relevant antigens, repeated immunization should not lead to loss of activity, and it must not give rise to significant titres of toxic and irrelevant antibodies. Thymocyte 'membrane' seems the best candidate so far studied and should provide a convenient starting material for further purification and extraction procedures.

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