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Solubilization by Butanol of Rabbit Cell-Antigens Associated with the Rejection of Transferred Lymph Node Cells. (30050)

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When rabbit lymph node cells are incubated *in vitro* with Shigella O antigen, washed and transferred to X-irradiated rabbits, agglutinins to Shigella appear subsequently in the sera of the recipient animals(1). Production of the antibody by the transferred cells can be prevented by prior injection of the prospective recipients with pooled rabbit leucocytes(2), or by incubation of the lymph node cells with rabbit anti-rabbit-leucocyte serum(3). Among the data indicating that the substance in the rabbit anti-rabbit-leucocyte serum causing suppression of transferred lymph node cells is an antibody was the observation that the suppressive antibody could be absorbed out of such sera by suspensions of whole lymph node cells. In further work, directed at purification and characterization of this antibody, it was found that it could also be absorbed from such suppressive sera by fragments of lymph node cells(4), prepared by differential centrifugation of suspensions of disrupted cells or of wash fluids of lymph node cells(5), and that from the cell-fragment-antibody complexes thus obtained the antibody could be eluted(4).

The availability of a specifically purified antibody which causes rejection of allogeneic transferred cells, and of a cell-fragment bearing the rejection-associated antigen, suggested studies on the solubilization of this antigen, in which the suppressive antibody would be used for detection of solubilized antigen. The extraction of such antigens by the use of Triton and snake venom phospholipase, based on a method described by Kandutsch and Stimpfling(6) for solubilization of mouse sarcoma antigens, has been reported elsewhere(7). A report is given here of the solubilization by butanol of rejection-associated antigens from cell fragments of normal rabbit liver or from Triton extracts of fragments of normal rabbit lymph node cells.

Materials and methods. Cells were prepared from lymph nodes and spleens of rabbits, and cell fragments were prepared from suspensions of these cells, as described elsewhere(5). For transfer of lymph node cells, cells prepared from popliteal and axillary nodes of 8 to 10 rabbits were washed, incubated at 37°C for 30 minutes with a Shigella-trypsin filtrate containing approximately 10^{-4} mg of solute per ml, washed again, and finally transferred in units of 2×10^8 to irradiated recipients, as described elsewhere (1). For preparation of rabbit anti-rabbit-leucocyte serum, rabbits were given 2 injections, at 5-week intervals, of 1.5×10^8 washed blood leucocytes pooled from 40 to 50 rabbits. The rabbits were bled 3 times between the 7th and 12th day after the injection, and the sera pooled.

Results and discussion. 1. *Spontaneous release of soluble antigen from normal rabbit liver cell fragments.* In an earlier study(4) cell fragments obtained by differential centrifugation of sonically disrupted lymph node cells had been found to react serologically with suppressive rabbit anti-rabbit-leucocyte sera and to absorb from such sera their effect of suppressing antibody formation by transferred lymph node cells. In the present study cell fragments of approximately similar centrifugal properties were obtained from rabbit liver cells as follows: Livers of normal rabbits

were minced, and the washed mince was placed in a Waring blendor with 2 volumes of buffered saline solution for 30 seconds. The blended preparation was centrifuged for 30 minutes at 3000 r.p.m. The sediment was discarded and the supernate was centrifuged at 16,000 r.p.m. for one hour. The pellet was collected, and the supernate, which was not clear (in contrast to the supernate of such centrifugation of sonically disrupted lymph node cells), was centrifuged at 30,000 r.p.m. for one hour. The sediment was collected and the clear supernate was discarded. The sediments collected at 16,000 r.p.m. and 30,000 r.p.m. were washed, in each case at the same centrifugal speed, and resuspended. The suspensions were tested by complement-fixation against suppressive rabbit anti-rabbit-leucocyte sera, and both were found to give positive reactions, the amount of antigenic material in the 16,000 r.p.m. pellet being 3 to 4 times that of the 30,000 r.p.m. pellet.

A chance observation made on a combined suspension of these two sediments was of interest. On 2 occasions such suspensions, originally very turbid, were found to have become clarified spontaneously in the course of a few days of storage at 4°C. Antigenic activity was sought in the clear solution, but only traces could be found, indicating that the enzymatic reaction which presumably caused the clarification of the cell-fragment suspension had broken down the antigenic determinants involved. Attempts were made to apply the hypothecated enzymatic reaction to the production of a soluble form of the antigen by incubation of cell-fragment suspensions at 37°C for various intervals of time. On centrifugation after these incubations the supernates did, in fact, contain soluble complement-fixing material, in concentrations increasing with increase in the period of incubation, but the relatively low yield of this material, and the appearance, after 4 hours of incubation, of anti-complementary material, sharply limited any possible application of this approach as a method of obtaining soluble antigen. It was, however, possible to obtain soluble antigenic material, in low concentration, in the supernates obtained by centrifugation of suspensions of liver cell-fragment incubated at 4°C for a few days. If the sediments of such centrifugation were resuspended for further storage at 4°C, centrifugation after a few days would again yield a supernate which contained soluble antigen, at about the same concentration. This could be repeated several times before the concentration of complement-fixing material in the successive supernates began to decline.

TABLE I. Solubilization of Serologically Active Material in Suspensions of Rabbit Liver Cell-Fragments on Incubation at 4°C.

Preparation	Collected at	Complement-fixation titer vs anti-leucocyte sera of: Pellet suspension	Supernate
	(r.p.m.)		
11	16,000	96	1.5
	30,000	24	3
12	16,000	64	.5
	30,000	32	3
13	16,000	192	.5
	30,000	64	3

The agent of this solubilization of the antigen was not studied. However, on centrifugation of each kind of pellet suspension after such a period of storage, and testing the sediment and supernate of each by complement-fixation it was found, as shown in Table I, that although there was a higher concentration of antigenic material in the 16,000-r.p.m. sediment of the pellet than of the 30,000-r.p.m. preparation, the soluble antigen was always in higher concentration in the supernate of the 30,000-r.p.m. pellet. Thus, on the hypothesis of an enzymatic reaction as the mechanism of solubilization of the antigen found in the supernates, although there was more substrate in the large cell-fragments, there was an indication of higher concentration of the enzyme in the higher speed pellet. Since a number of enzymatic activities have been found to be associated with ribosomes, and since the sedimentation of the bulk of the ribosomes of fragmented cells requires centrifugal fields in excess of those used here, it may be that the solubilization of the antigen encountered here was caused by enzymes of ribosomes contaminating these cell-fragment pellets, such contamination being of greater degree in the prepa-

ration obtained with the higher centrifugal field.

It is of interest in this connection that soluble cell-antigen associated with transplantation effects has been encountered in other situations, as in the finding by Davies(8) of soluble antigen having strain specificity in the ascitic fluid of mice with ascites tumor cells. It may be that the same mechanism is involved in both situations.

2. *Solubilization of the antigen from liver cell fragments by butanol.* A number of attempts to free the antigen from its original association with cell fragments, such as exposure to lipases(9,10) or to deoxycholate, which has been used for dissociation of cytochrome oxidase from mitochondria by Watson and Siekevitz(11), or polypeptidylization with N-carboxy-α-alanine, as was found by Sela *et al*(12) to solubilize gliadin, produced very little or no soluble antigenic material. An effective method was found in the use of butanol, originally described by Morton(13) and since then used for dissociation of lipoprotein complexes in a number of situations(14).

In initial experiments on the dissociation of a soluble antigenic material from the liver cell fragments, a suspension of washed cell fragments was divided into 4 parts. Each was chilled and placed in a beaker which was kept in an ice bath while being stirred with a magnetic stirrer. Butanol was added very slowly so that the addition of 20% of the volume of the suspension required about 30 minutes. To 3 of the beakers butanol was added up to 5%, 10%, and 20%, respectively, of the volume of cell-fragment suspension, and mixing was continued until one hour after the beginning of the addition of butanol. The 3 preparations, and the control portion, were then centrifuged for 30 minutes at 16,000 r.p.m. The supernate, or, in the case of the 2-phase system encountered with 20% of butanol, the lower layer of liquid, was removed and dialyzed overnight against cold distilled water, after which the dialyzed supernate and the washed, resuspended pellet were examined by complement-fixation against suppressive antisera. Table II shows that the treatment with butanol caused the release of soluble antigen, and

TABLE II. Solubilization of Serologically Active Material by Butanol.

Butanol vol (%)	Complement-fixation titer vs anti-leucocyte sera of: Butanol extract	Resuspended sediment
0	–	48
5	3	24
10	4	12
20	6	6

that with addition of an increasing volume of butanol there was an increasing concentration of soluble antigen in the extract, up to the maximum volume used, 20%. Also, with increasing concentrations of butanol there was loss of residual antigenic activity in the insoluble residue, so that the pellet recovered from butanol-saturated solution retained only a small fraction of the original activity of the cell-fragment preparation, even after allowing for the amount of antigen solubilized.

The antigenic material solubilized in this way was soluble in distilled water, could be lyophilized without observable loss of activity, and was easily excluded by Sephadex G-25. (The separation from butanol could be carried out by this means alternatively to dialysis.)

In a preliminary chromatographic examination a butanol-solubilized preparation was applied to a column of DEAE-cellulose in PO_4 buffer, 0.01 M, pH 7.8, and the adsorbed material was eluted in 2 steps of increasing concentration of NaCl, 0.25 M and 0.5 M, in the starting buffer. Successive 3-ml portions of effluent from the column, before the elution and after each step of NaCl, were examined for optical density (OD) at 280 mμ and complement-fixing activity. It was found, as shown in Fig. 1, that a small amount of OD-positive material was not adsorbed to the cellulose, and that of the protein adsorbed the greater part was eluted with 0.25 M NaCl and most of the remainder (85% altogether) was eluted at 0.5 M NaCl. Of the serologically active material, it can be seen that all of it was adsorbed to the cellulose and all was eluted in the step at 0.25 M NaCl. Within the material eluted at this concentration of NaCl, a comparison of the location of the peaks of OD and com-

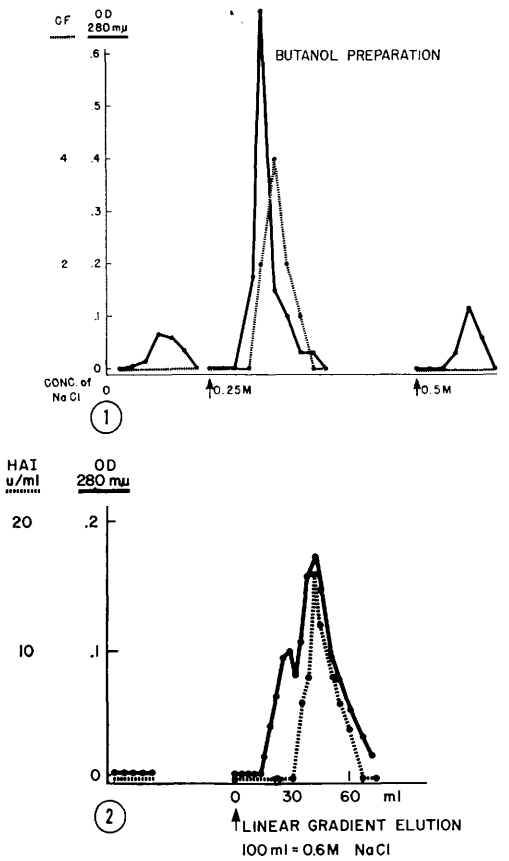

FIG. 1. Examination of butanol extract of rabbit liver cell-fragments by column chromatography (DEAE-cellulose; starting buffer pH 7.8 phosphate, 0.01 M). OD at 280 mμ and complement-fixation *vs* anti-leucocyte serum of successive 3-ml portions of unadsorbed material, material adsorbed and eluted on application of 0.25 M NaCl in the starting buffer, and material eluted with 0.5 M NaCl.

FIG. 2. Examination by column chromatography of Triton-extracted lipoprotein of cell fragments from rabbit lymph node and spleen. Same starting conditions as in Fig. 1, then elution with a linear gradient increase of NaCl in the starting buffer. For successive 3-ml portions of the column eluate are shown the OD at 280 mμ and inhibition of the agglutination of antigen-coated RBC by cell-suppressive rabbit anti-rabbit-leucocyte serum.

plement-fixing activity suggests the presence of inactive protein, which is apparently eluted from the adsorbant at a lower concentration of salt than is the active antigen.

3. *Solubilization of the antigen by butanol from a Triton-extract of cell fragments.* The first application of the method of solubilization described above was to preparations of liver cell-fragment suspension which had been stored for several weeks at 4°C for repeated extractions of the spontaneously solubilized antigen, as described above. On extending the application of the butanol method to more recently prepared suspensions, yields of preparations were obtained in which the soluble antigen was either of much lower concentration or apparently entirely absent. Further, there were variations in the yield from preparations even of approximately the same period of storage at 4°C. It was considered that an enzymatic preparatory step might be required, perhaps a partial dissociation of a lipoprotein complex from the entire cell fragment, in order to make possible the dissociation by butanol, and that this reaction, proceeding slowly at 4°C, might have been of sufficient extent during the longer periods of storage of the liver cell fragments to cause the preparative dissociation for the butanol effect. Accordingly, the butanol procedure was applied to the water-insoluble lipoprotein extracted by Triton from cell fragments of liver or of spleen and lymph node of the rabbit. In the case of material from both cell types the solubilization was successful, but since the yields per gram were 10 or more times as great from spleens and lymph node as from liver (in keeping with a similar ratio of serologic activity of the cell-fragment suspensions themselves), the preparation from Triton-extracts of cell fragments from spleen and lymph node will be described:

Lyophilized suspensions of cell-wash pellet (5) pooled from the spleens and lymph nodes of many rabbits were extracted with Triton and the extract was treated with acetone (6, 7). The acetone precipitate was extracted with buffered saline solution, and the residue was lyophilized. This water-insoluble material, which was about 20% of the weight of the lyophilized cell-fragment, was suspended in saline solution at 20 mg/ml, and butanol was added in 20% of the volume of the suspension as described above. After removal of butanol from the aqueous phase by dialysis, it was lyophilized, with a yield of approximately 1.5% of the original weight of lyophilized cell-fragment.

The butanol-extracted material did not fix complement with anti-leucocyte sera, but

serologic evidence of the presence of the cell antigen was obtained in adsorption-hemagglutination tests. As was reported elsewhere, the antigenic material solubilized from Triton extracts of rabbit spleen cell fragments by snake venom phospholipase could be adsorbed to tannic-acid-treated sheep RBC, and such cells were agglutinated by suppressive rabbit anti-rabbit-leucocyte sera. In the present experiments it was found that this agglutination could be inhibited by prior incubation of the anti-leucocyte serum with the butanol-extracted material. Further, the butanol-solubilized antigen could itself be adsorbed to tannic-acid-treated RBC, with subsequent agglutination by anti-leucocyte sera.

The antigen solubilized by butanol from the Triton-extracted lipoprotein was, again, found to be water soluble and to be excluded by Sephadex (in this case even by Sephadex G-200). For chromatographic examination a preparation was applied to DEAE-cellulose under the same conditions as above. As can be seen in Fig. 2, all the protein was adsorbed to the column. On linear gradient elution with NaCl in the starting buffer, at least one component other than the serologically active antigen could be discerned, its presence being more clearly indicated than in the butanol extract of untreated liver cell-fragment, presumably because of the gradient, rather than stepwise, mode of elution.

4. *Relation of butanol-solubilized antigen to rejection of allogeneic transferred lymph node cells.* The serologic observations referred to above gave evidence of the presence in the butanol extract of solubilized antigen, by its serologic reaction with an antibody in suppressive sera. To test whether this antigen was the one associated with the rejection of transferred lymph node cells, 5 rabbits were injected twice, at an interval of 3 weeks, with the butanol-extracted antigen, each injection consisting of 5 mg of the lyophilized preparation dissolved in saline solution, the solution being emulsified with an equal volume of Bayol and Arlacel A, as suggested by Freund(15). One week after the second injection the rabbits were bled and serum prepared from these samples was pooled. This serum pool was tested for activity in suppressing transferred lymph node cells, by obtaining such cells from donor rabbits, incubating these with *Shigella* antigen, then incubating them with 2 volumes of the pooled serum, at various concentrations, before transfer to recipient rabbits. From titrations of agglutinins to *Shigella* in the sera of the recipient rabbits at appropriate intervals thereafter, the maximal titer was obtained, as a measure of the amount of antibody produced by the lymph node cells transferred. Geometric means of the maximal titers were calculated for each group of rabbits given cells which had been incubated with a given concentration of the antiserum, and the difference between this mean and the mean for control recipients is shown for 2 such experiments, in power of 2, in Table III. It can be seen in the Table that incubation of the transferred cells with the antiserum at 1:16 caused no reduction in antibody production by those cells. However if the antiserum was used at 1:8 the maximal titer was reduced by 3 to 3.4 powers of 2, or to about 15% of the control level, and at 1:4 of the antiserum the reduction was by 2^4, or to about 6% of the mean value of recipients of untreated cells. The injection of rabbits with the butanol-solubilized antigen had, then, clearly given rise to the production of antibodies causing suppression of function of transferred allogeneic lymph node cells.

TABLE III. Suppression of Antibody Formation by Transferred Lymph Node Cells on Incubation of the Cells with Pooled Serum of Rabbits Injected Twice with Butanol-Solubilized Antigen.

Concentration of serum for incubation with lymph node cells	Reduction of geometric mean maximal antibody titer in sera of recipients from control level ($\log_2$)	
1:2	4.8	
1:4	4.0	4.0
1:8	3.0	3.4
1:16		.3

Summary. Studies have been carried out on the solubilization of the rabbit cell-antigen associated with rejection of transferred allogeneic lymph node cells. Using as a preliminary criterion serologic reaction *vs* cell-suppressive rabbit anti-rabbit-leucocyte sera, soluble antigen has appeared in low concentration in suspensions of liver cell-fragments from normal rabbits kept for some days at

4°C. The antigen was solubilized by the use of butanol at 20% by volume from suspensions of such liver cell-fragments, if the suspensions were previously maintained at 4°C for some time. Butanol extraction could also be applied to Triton-extracted lipoprotein of cell-fragments obtained from normal rabbit lymph node and spleen with serologically active material obtained in solution in about 10 times the yield obtained from liver, relative to mass of tissue. When the butanol-extracted soluble antigen from rabbit lymph node was injected twice into normal rabbits, the sera of the latter showed the property of suppressing antibody formation by transferred lymph node cells on incubation with the cells *in vitro* prior to transfer.

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Beta-hydroxyacyl Dehydrogenase in Human Arterial Tissue.* (30051)

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Beta-hydroxyacyl dehydrogenase is an enzyme of the fatty acid cycle which catalyzes the following diphosphopyridine nucleotide (DPN) dependent reaction:

Acetoacetyl-Coenzyme A + DPNH + H^+ ⇄ Beta-hydroxybutyryl-Coenzyme A + DPN^+

Because beta-hydroxyacyl dehydrogenase is involved in the biosynthesis and catabolism of lipids, it was considered important to study its activity in human arterial tissue. The enzyme assays were made separately on normal and arteriosclerotic tissue. For comparative purposes, a limited number of venous samples was included in the investigation.

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Materials and methods. Vascular samples were obtained fresh at autopsy from subjects ranging in age from 0 to 85 years and were immediately frozen. A total of 215 specimens was included in the study. The determinations of beta-hydroxyacyl dehydrogenase activities were made by a modification of the procedure described by Michal and Bergmeyer(1).

The acetoacetyl-coenzyme A substrate was prepared fresh daily through treatment of coenzyme A (Sigma Chemical Co., St. Louis) with thioglycolic acid and diketene(2). Ten % aqueous homogenates of intima-media layers of the vascular samples were made at 4°C with a Kontes Duall type tissue grinder; the homogenates were subsequently centrifuged at 25,000 r.p.m. and the supernatants immediately used for enzyme determination.