Changes in Lipid Pattern of HeLa Cells Exposed to Immunoglobulin G and Complement

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1. Immunoglobulin G was isolated from sera of non-immunized rabbits or rabbits immunized with whole HeLa cell homogenate. The anti-HeLa immunoglobulin G and its Fab fragment precipitated the particulate 400 000g-min. fraction of HeLa cell homogenate. 2. Immunoglobulin G from immunized or non-immunized rabbits and fresh or inactivated complement were added to HeLa cell cultures. Changes in the cell count and cellular contents of DNA, RNA, protein, total and individual phospholipids, cholesterol (and esters) and ganglioside were followed. 3. Addition of immunoglobulin G from non-immunized rabbits and guinea-pig serum (complement) caused a transient increase in DNA followed by a permanent increase in RNA, protein, dry weight and number of cells per culture. 4. Addition of anti-HeLa immunoglobulin G and active complement caused an increase in the cellular content of cholesterol, total phospholipids, lysophosphatidylcholine and lysophosphatidylethanolamine greater than the increase of the controls and a decrease in the molar percentages of phosphatidylcholine and phosphatidylethanolamine as compared with the controls. 5. The cholesterol/phospholipid ratio remained constant. 6. The appearance of lysophosphoglycerides was transient, reaching a maximum 3 hr. after addition of anti-HeLa immunoglobulin G. 7 The content of lysophosphoglycerides in HeLa cultures exposed to immunoglobulin G from non-immunized rabbits ranged from 50% to 30% of the values obtained from cultures exposed to the anti-HeLa immunoglobulin G and complement. 8. The changes in the lipid pattern of the HeLa cells were associated with the appearance of juxta-nuclear vacuoles in cells, but were apparently not specifically related to the presence of active complement.

In the presence of complement, antibodies to nucleated cells initiate membrane disarrangements by unmasking phospholipids, forming pits and holes (diam. 80–100 Å), and activating lysosomal enzymes (Goldberg & Green, 1959; Bitensky, 1963; Dumonde, Bitensky, Cunningham & Chayen, 1965; Dingle, Fell & Coombs, 1967; Benedetti & Emmelot, 1968). During this process, the membrane forms filaments (Benedetti & Emmelot, 1968); and K^+ (Green, Barrow & Goldberg, 1959), amino acids and enzymes (Dingle *et al.* 1967) are liberated.

The aim of the present investigation was to elucidate the changes in the lipid pattern of HeLa cells exposed to antibodies, since no such studies of nucleated cells are yet available.

MATERIALS AND METHODS

HeLa-cell cultures. HeLa cells (Microbiological Associates, Bethesda, Md., U.S.A.), were cultured as monolayers in Hanks balanced salt solution (Hanks solution) (Paul, 1961), enriched with 10% (v/v) of horse serum (Leo Pharmaceutical Products, Ballerup, Denmark), 0.2% (w/v) of lactalbumin

hydrolysate (Difco Laboratories, Detroit, Mich., U.S.A.), 0.07% (w/v) of yeast extract (Difco), 2.05 mm-glutamine, 5.56 mm-glucose, 0.24 mm-penicillin and 0.86 mm-streptomycin; $NaHCO_3$ was added to maintain a pH of 7.38. Stock cultures were grown in 1 l. Roux flasks. For immunological experiments, 10^6 /ml. cells were transferred to 2 cm. $\times$ 12 cm. glass Petri dishes.

Since our initial studies (Güttler & Clausen, 1969) showed the optimum growth rate to be dependent on oxygen partial pressure (i.e. that 40% O_2 retarded the growth and low oxygen partial pressure promoted glycolysis), all cultures were incubated at $36 \pm 0.5^\circ$ in a water-saturated atmosphere of $O_2 + CO_2 + N_2$ (25:5:70). Media were changed every second day.

The growth of cells was followed by time-lapse cinematography. For this purpose the cells were grown in 30 ml. T-flasks (Falcon).

Complement. Fresh guinea-pig blood was delivered by Statens Seruminstitut, Copenhagen S, Denmark. After clotting for 2 hr., the serum was isolated by centrifugation and sterilized by filtering. It was stored in sealed glass ampoules at -60° .

Rabbit serum. This was obtained from healthy non-immunized rabbits (wt. 2–4 kg.) of both sexes, maintained at Statens Seruminstitut.

Reagents and standard mixtures. (a) Chemicals. Amino acids, vitamins and carbohydrates for tissue-culture media were obtained from British Drug Houses Ltd., Poole, Dorset; penicillin, streptomycin and mycostatin were from Leo Pharmaceutical Products. A.R.-grade chloroform, methanol, HCl, acetic acid, aq. NH_3 (sp.gr. 0.88), HClO_4 , H_2SO_4 , silica gel H, ninhydrin spray reagent (0.1%) and resublimed iodine were from E. Merck A.-G., Darmstadt, Germany.

1,1,2-Trichloroethane (purum, b.p. 111–114°) was obtained from Fluka A.-G., Buchs SG, Switzerland. Redistillation of this fluid did not decrease blank readings in the determinations of cholesterol.

3,5-Diaminobenzoic acid dihydrochloride used for DNA determinations (purum, m.p. 236–238°; Fluka A.-G.) was recrystallized before use (Hess & Rolde, 1964).

(b) Reference mixtures of phospholipids. Lysophosphatidylcholine (10 μg .), lysophosphatidylethanolamine (10 μg .) (both from Sigma Chemical Co., St Louis, Mo., U.S.A.), sphingomyelin (10 μg .), phosphatidylserine (10 μg .), phosphatidylethanolamine (20 μg .) and phosphatidylcholine (25 μg .) (all from Koch–Light Laboratories Ltd., Colnbrook, Bucks.) per 5 μl . of chloroform–methanol (2:1, v/v) were used as reference markers in t.l.c. A total of 4 μg . of lipid marker P/5 μl . sample of the reference mixture was used.

(c) Ashing mixture. This was 3M- H_2SO_4 in 28% (w/v) HClO_4 .

(d) Reducing reagent. This was made according to Fiske & Subbarow (1925), with amino-2-hydroxynaphthol-4-sulphonic acid (E. Merck A.-G.) and sodium bisulphite (Baker Analyzed reagent; J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.).

Cleaning and sterilization. A 10% (v/v) solution of the anionic detergent 7X (Linbro Chemical Co. Inc., New Haven, Conn., U.S.A.) in deionized water was used for cleaning all glassware. Glassware for tissue culturing was rinsed again in triple glass-distilled water and sterilized (160° for 3 hr.). Glassware for analytical use, including constriction pipettes (the Lang–Levy type) and plates for t.l.c. were rinsed further in H_2SO_4 saturated with $\text{K}_2\text{Cr}_2\text{O}_7$, followed by four rinsings in running tap-water and four in deionized water. Sera, glucose, amino acids, vitamins, NaHCO_3 and antibiotic solutions were sterilized by filtration through Jena 5g. glass filters (pore diam. 1.7–3.0 μm .). Stock media (i.e. without the above-mentioned solutions added) were autoclaved at 120° (10 min.).

Immunization. HeLa cells were washed four times in Hanks solution and freeze-dried. The dry weight was measured and the material was suspended in sterilized water to give a total protein content of 3.0 mg./ml.: sodium deoxycholate (1 mg./mg. of protein) was added to the antigen suspension and the mixture homogenized in an equal volume of Freund's incomplete adjuvant (Difco). At 8 days before immunization of the rabbit, 0.5 ml. of Freund's complete adjuvant (Difco) was injected intramuscularly in each hind leg and 1.0 ml. intraperitoneally. Then 50 μl . of antigen solution was injected intracutaneously in the hind foot-pads and 1.0 ml. intraperitoneally; this step was repeated 3 weeks later without Freund's adjuvant. Booster injections of 10^5 cells, washed as described above and suspended in sterilized Hanks solution, were given intravenously 7 days before a blood sample was taken. Blood (25 ml.) was obtained by heart puncture twice monthly (Clausen, 1969).

Preparation of particulate HeLa-cell antigens. Approx. 10^7

HeLa cells were washed four times in Hanks solution, resuspended in 2 ml. of the same solution and disintegrated by ultrasound (Branson Sonifier; 35 w for 1.0 sec.). The nuclear fraction was obtained from the crude homogenate by centrifugation at 600 g_{max} . for 10 min. at 4°. From the 6000 g_{min} . supernatant, a crude mitochondrial and lysosomal fraction was obtained by centrifugation in a Sorvall RC-2 centrifuge at 20000 g_{max} . for 20 min. at 4°. The supernatant from this step was finally centrifuged in a Beckman L-2 ultracentrifuge (rotor no. 25.2) at 100000 g_{max} . for 1 hr. at 4° to collect the ribosomal fraction. All particulate fractions were resuspended in 400 μl . of 0.5% (v/v) Triton X-100, containing 1% (w/v) of sodium deoxycholate, and disintegrated by treatment with ultrasound twice at 45 w for 2 sec. Finally, the supernatants (1.2–2.4 g of total protein/100 ml.) were obtained by centrifugation at 20000 g_{max} . for 20 min. at 4° and subjected to immunodiffusion and immunoelectrophoresis.

*Isolation of the electrophoretically slow-moving rabbit IgG.** The slow-moving IgG of normal and immunized rabbits was isolated from whole serum on DEAE-cellulose (Sigma) with a continuous gradient of 0.03–0.20 M-potassium phosphate buffer, pH 8.0 (Fahey & Horbett, 1959). The initial protein peak, appearing in the effluent before the major change in molarity, was collected. Fractionation was performed on 40 cm. $\times$ 2.6 cm. (Quickfit) columns. Packing was performed with a wet settle volume/total volume ratio of 1.4. When 1.7 g. of whole serum protein was fractionated on this column, 0.18 g. of immunoelectrophoretically pure IgG with low electrophoretic mobility was obtained. The void volume was 100 ml. and the effluent volume was 30 ml. An automatic set-up at 4° consisting of an LKB Radial Rac fraction collector, an LKB Uvicord spectrophotometer and a Radiometer conductivity meter (4°) connected to an LKB six-channel d.c. recorder was used. The effluents of the initial peak were vacuum-dialysed against Hanks solution (Clausen, 1969). The instruments used were sterilized. Penicillin (100 μg ./ml.), streptomycin (50 μg ./ml.) and mycostatin (50 units/ml.) were added to buffers and Hanks solution.

Zone electrophoresis. This was carried out in agar gel (Difco Noble Agar) (1%, w/v) in sodium barbiturate buffer, pH 8.4, as described by Wieme (1959).

Immunoelectrophoresis. This was standardized as indicated by Clausen (1969). A 1 cm.-thick agar gel in 0.05 M-sodium barbiturate buffer, pH 8.6, was applied on glass micro-slides (76 mm. $\times$ 26 mm.).

Immunoelectrophoresis was used to demonstrate the antibody activity of chromatographic effluents and to characterize the purity of the rabbit IgG isolated from serum by column chromatography (see above). The first was done by applying 2.0 μl . of antigen sample (HeLa-cell homogenate; see above) (2 g./100 ml.) to the well, with 80 μl . of antiserum effluent (3 g./100 ml.) in the trough. To assay the purity of the rabbit IgG, 2.0 μl . of globulin (7 g./100 ml.) was applied in the electrophoresis. In the following immunodiffusion, 80 μl . of anti-rabbit antiserum from donkeys (Behringwerke A.-G., Marburg, Germany) diffused towards the electrophoretic fractions. A pooled rabbit serum (2.0 μl .; 7 g./100 ml.) was always used as standard in a well opposite to that of the sample.

The electrophoretic step was performed at 8 v/cm, for

*Abbreviation: IgG, immunoglobulin G.

1½ hr. at 30°; immunodiffusion followed for 16 hr. at 22°. The slides were washed in 0.15M-NaCl for 2 days and in water for 2 hr. Once dry, the pattern was detected after protein staining with Amido Black (Clausen, 1969).

Double immunodiffusion in agar. This technique (standardized as indicated by Clausen, 1969) was employed to study the immunological specificity of the chromatographic fractions from sera of immunized and non-immunized rabbits, and to assess against which particulate fractions antibodies were produced in the rabbits. The immunodiffusion was performed in agar slides as used for immunoelectrophoresis. The circular-well technique, with four holes 1 cm. apart and 1 cm. from the central well, was used. Antigen samples (2.0 µl.; 2 g./100 ml.) were applied peripherally. In the central well the IgG fraction (2.0 µl.; 7 g./100 ml.) was applied. Immunodiffusion and interpretation of results were performed as described by Clausen (1969).

Evaluation of antibody titre. The antibody titre of the chromatographic fractions was evaluated by quantitative radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) in plastic Petri dishes (5 cm. diam.). Agar gel (2%, w/v) in 0.05M-sodium barbiturate buffer, pH 8.6, was melted in a Roux flask. After cooling to 45°, an equal volume (1.2 ml.) of whole crude HeLa-cell antigen suspension (2 g. of protein/100 ml.) was added and mixed, and the solution poured into the dishes in a 1 mm.-thick layer. After gelling, 1 mm. holes were sucked in the gel. Then 1.5 µl. of slow-migrating IgG chromatographic fractions (0.64–3.64 g./100 ml.) was applied in the gel in increasing concentration of total protein. By relating the diameter of the leading edge of the immunoprecipitate after immunodiffusion for 2 days to the total protein content of the sample, a rectilinear standard curve was obtained. On the basis of this, the antibody titre was in all experiments standardized to give a 3.5 mm.-diam. area of immunodiffusion/37.8 µg. of IgG fraction applied to the hole.

Characterization of antibody. The IgG consists of two main fragments obtainable by cleavage with papain (Porter, 1959), i.e. the Fab and the Fc pieces. The former contains the antibody site, the latter the receptor for cell surfaces. Since both pieces may combine with particulate fractions, Clausen (1969) stresses the necessity for specifying which piece precipitates the antigen. The precipitating specificity of the Fab and Fc pieces of the IgG was therefore studied. To do this, the isolated rabbit IgG of immune serum (diluted two-fold with 0.15M-NaCl) was prepurified by precipitation from whole rabbit serum with 2.0M-(NH₄)₂SO₄, pH 6.8, at 22° for 3 hr. The precipitate obtained by centrifugation was washed twice by resuspension in 2.0M-(NH₄)₂SO₄ and isolation by centrifugation. The precipitate was finally dissolved in an appropriate volume of 0.1M-sodium phosphate buffer, pH 7.0, and 0.01M-cysteine, 2 mM-EDTA (disodium salt) and papain (1%, w/v) were added (Porter, 1959). Digestion was allowed to proceed for 16 hr. at 37°. The digested fraction was dialysed against water and finally against 0.15M-NaCl. The Fab and Fc fragments were finally separated on Sephadex G-25 by applying 100 mg. of digested IgG to a column (45 cm. × 2.0 cm., bed height 30 cm., free height of eluting solution 10 cm.) equilibrated with 0.15M-NaCl. Fractions (3 ml.) were collected with the set-up described above. Elution with 0.15M-NaCl was continued until the amount of protein emerging from the column had fallen to zero (210 ml.). A major protein peak corresponding to the elution volume of 51 ml. appeared to-

gether with four minor peaks at 70, 110, 140 and 180 ml. The major peak was asymmetric, its ascending line forming a plateau at 40 ml. This part was collected separately; the remaining fractions of the major peak and the fractions of the minor peaks were pooled separately. The pooled fractions were concentrated by freeze-drying followed by dialysis to give a total protein content of 1 g./100 ml.

Immunoelectrophoresis revealed pure Fc fragment to be in the first 40 ml. and in the 170–210 ml. fraction. The Fab fragment was found together with the Fc fragment in the three intermediate fractions. Fab fragment was differentiated from Fc fragment by the fact that the first fraction on immunoelectrophoresis migrates more rapidly to the cathode than the Fc fragment (Heremans, 1960). The antigen-combining capacity was localized to the Fab fragment and not the Fc fragment by immunodiffusion and immunoelectrophoresis.

Harvesting of cells and counting. Before harvesting, medium was drawn off the cells and the monolayer was washed twice in Hanks solution (4°). The cells were harvested in 8 ml. of solution with a Teflon-coated glass rod and then centrifuged (600g for 10 min. at 4°) and resuspended in 8 ml. of solution. To 200 µl. of the cell suspension, 200 µl. of 0.1M-citric acid was added. After incubation for 1 hr. (37°), the tubes were shaken vigorously and 100 µl. of Crystal Violet solution (1 mg./ml. of 0.1M-citric acid) was added. The stained nuclei were counted in a haemocytometer.

Extraction of lipids. An initial study was made on the yield of lysophospholipids by two different extraction procedures. One extraction method involved solubilization of lipids by the procedure of Folch, Lees & Sloane-Stanley (1957), from material that had been immediately deep-frozen (–80°) and then freeze-dried. The other method involved a direct extraction of the lipids from wet material with a chloroform-methanol mixture at –30° (as below). A 10% higher yield of lysophosphatidylcholine was obtained by the first method although the yield of total phospholipids was identical in the two methods. This difference could be due to formation of lyso compounds during the freeze-drying process. The wet extraction was therefore preferred.

The harvested and washed cells were centrifuged in tapered 16.5 mm. × 100 mm. Pyrex centrifuge tubes (7000g for 15 min. at 4°). The Hanks solution was sucked off and 1 ml. of chloroform-methanol (1:2, v/v) (–30°) was added. The contents were homogenized at 200 rev./min. (4°) with a glass tissue-grinder pestle (size B; Kontes Glass Co., Vine-land, N.J., U.S.A.). The tubes were filled with N₂, sealed and submitted to temperature shock for 90 min. at –70° and then for 30 min. at 4°, three times in succession. Then the supernatant extracts were isolated by centrifugation (7000g for 20 min. at 4°), and extraction repeated. The extracts were pooled and stored under N₂ (–30°). A third extraction was performed by keeping the residue for 16 hr. under N₂ (4°). The combined extracts (3 ml.) were centrifuged at 20000g for 30 min. at 4°. The clear supernatant was transferred to balanced tubes just before partitioning, dried in a centrifuge-evaporator (Albers & Lowry, 1955) and balanced.

Separation of lipids. The dried extracts (600–800 µg.) were dissolved in 80 µl. of chloroform-methanol (1:1, v/v) (4°). A 15 µl. sample was used for determination of cholesterol (see below), 15 µl. for the determination of lipid P and 20 µl. for t.l.c. All assays were done four times for each extract.

Determination of lipid phosphorus, sialic acid and proteo-lipid. The 15 μ l. sample for determination of lipid P was further diluted with 1 ml. of chloroform-methanol (2:1, v/v) and 200 μ l. of 15 mM-KCl was added, followed by mixing on a Whirlimixer (Scientific Industries Ltd., London). The tubes were kept for 16 hr. at 4°, after which the upper phases were isolated. To the lower phases was added 200 μ l. of theoretical upper phase (chloroform-methanol-0.5 M-NaCl in tris buffer, pH 8.8; (3:48:47, by vol.) (Webster & Folch, 1961). The mixtures were gently mixed and kept for 16 hr. The upper phase was isolated and combined with the previous one, dried in the centrifuge-evaporator and assayed for sialic acid (Hess & Rolde, 1964) and protein (Lowry, Rosebrough, Farr & Randall, 1951). The chloroform (lower) phase was dried, redissolved in 30 μ l. of chloroform-methanol (1:1, v/v) and divided into four 5 μ l. samples for determination of lipid P.

Determination of cholesterol and cholesteryl esters. Cholesterol was determined fluorimetrically (Albers & Lowry, 1955). Lipid extract (15 μ l.) was redissolved in 30 μ l. of chloroform-1,1,2-trichloroethane (2:1, v/v). Chloroform-trichloroethane was preferred since chloroform-methanol gave a sigmoid standard curve. Portions (5 μ l.) were dried under N₂ and then dissolved in 150 μ l. of 1,1,2-trichloroethane acetic anhydride (5:1, v/v). Then 7 μ l. of 98% (v/v) H₂SO₄ was added and the contents of the tube were immediately mixed. After reaction at 22° for 90 min., 1 ml. more of trichloroethane-acetic anhydride (5:1, v/v) was added, and the fluorescence read (Photovolt 540 fluorescence meter; Photovolt Corp., New York, N.Y., U.S.A.). [The 546 nm. mercury line was used as the exciting source, isolated with a Jena (Jena Glaswerk, Mainz, Germany) 550 nm. interference filter (half-width 17 nm., peak transmittance 47%).] The secondary filter was a Jena double-bandpass filter with 25% transmittance at 590 nm. Six duplicate cholesterol standards [Sigma grade: 99%, stock no. CH-S; according to Williams & Witter (1965) it was found that the Sigma preparation employed is 99% pure] and reagent blanks were included. The molar content was obtained from the values in micrograms by multiplication by 2.59. The recovery of 5 μ g. of cholesterol with this method was 93.3–98.7%. With 6.8 μ g. of cholesterol the analytical error was 3.5%.

Cholesteryl esters were determined after precipitation by digitonin (16 hr. at 4°) [50 mg. of digitonin krist. (E. Merck A.-G.) in 10 ml. of 50% (v/v) ethanol] (Glick, 1961). The cholesterol contents of the precipitates obtained after centrifugation at 20000g for 30 min. were measured as described above, with the ethanolic digitonin solution as blanks [cholesteryl acetate (Sigma, stock no. CH-SA) was used as standard].

Determination of lipid-soluble N-acetylneuraminic acid and protein. The combined upper phases (see above) were dried slowly in the centrifuge-evaporator and redissolved in 350 μ l. of redistilled water. Duplicate samples (100 μ l.) were assayed for N-acetylneuraminic acid by the fluorimetric method of Hess & Rolde (1964). Duplicate samples (50 μ l.) were used for protein determination (Lowry *et al.* 1951).

Separation and quantitative analysis of phospholipids. A two-dimensional separation of the phospholipids was performed on silica gel H (E. Merck A.-G.) (as below). After digestion of the lipid spots, the individual phospholipids were determined by their P content.

Silica gel H (200 g.) was washed twice by chloroform-methanol (4:1, v/v), applied as a 0.25 mm. layer on 20 cm. $\times$ 20 cm. glass plates (Stahl, 1962) and activated for 2 hr. at 110°. Before use, the t.l.c. plates were soaked in chloroform-methanol (4:1, v/v) overnight (Skipski, Peterson & Barclay, 1964) and activated at 110° for 1 hr. immediately before use. The chromatography was performed in 10 cm. $\times$ 25 cm. $\times$ 25 cm. glass tanks lined internally with Whatmann 2MM filter paper.

Since lysophospholipids are partly soluble in water (Marinetti, 1962), t.l.c. was performed before washing of the lipid extracts. A 10 μ l. sample containing an average of 25–75 nmoles of phospholipid (0.8–2.3 μ g. of lipid P) was applied on the lower edge during development with a Tusomo microlitre syringe as a 10–12 mm.² spot, 3 cm. from one corner. A mixture of reference phospholipids was applied 3 cm. from the opposite lower corner. The plate was then subjected to ascending chromatography for 16 cm. in solvent I [chloroform-methanol-aq. 33% (w/v) ammonia (13:7:1, by vol.)]. The plate was dried under N₂ (60° for 3 min.) and then exposed to the fumes of acetic acid for 20 min. The plate was then developed from the left edge for 10 cm. with solvent II [chloroform-methanol-acetic acid-water (25:15:4:2, by vol.)], dried under N₂ (60° for 3 min.) and finally developed from the right edge for 10 cm. with solvent II.

The red-violet spots appearing 5–10 min. after spraying with ninhydrin were outlined with the tip of a capillary glass tube. The plate was then exposed to iodine vapour for 3–15 min. and the developed spots were immediately outlined. The outlined spots were identified by comparison with reference phospholipids. Each spot was sucked directly into a 20 ml. Pyrex digestion tube, by using the aspiration apparatus of Rouser, Siakos, Fleischer & Simon (1966). Six spots of a control sample, containing 0.2, 0.5 and 1.0 μ g. of P (made from a stock solution of KH₂PO₄ in water), and two spots each containing 25 μ g. of phosphatidylcholine were included. Blank values were obtained by sucking blank areas of the t.l.c. layer of similar sizes. Then 250 μ l. of ashing mixture was added and the tubes were heated in a sand bath for 2 hr. Loss of HClO₄ (Rhee & Dugan, 1967) was prevented during this procedure by stepwise increase in temperature: within the first hour from 150° to 170° and during the next hour from 170° to 205°. At this time the solution (180–200 μ l.) had become colourless and the SO₃ had evaporated. At this point the ashing was stopped to prevent formation of pyro- or meta-phosphates (Fiske & Subbarow, 1925) and to keep the concentration of H₂SO₄ in the reaction volume at about 0.25–0.35 M (Fiske & Subbarow, 1925). The P content was determined by a microadaptation of the method of Bartlett (1959), with 1–500 ml. of freshly prepared 0.22% (w/v) ammonium molybdate in redistilled water and 50 μ l. of reducing reagent. The tubes were capped with glass stoppers, mixed vigorously and placed in a boiling-water bath for 7 min. After cooling and removal of the suspended adsorbent by centrifugation, the extinction of the samples was read at 820 nm. in a Beckman DU spectrophotometer equipped for sample volumes of 200 μ l.

For expression of results as nmoles of phospholipid, the values for P were multiplied by the following factors: phosphatidylcholine, 32.3; phosphatidylethanolamine, 31.6; phosphatidylinositol, 30.4; phosphatidylserine, 32.4; sphingomyelin, 32.4; lysophosphatidylcholine, 32.3; lysophosphatidylethanolamine, 32.3 (Debuch, 1964). The ana-

lytical error in the range of 200–500 ng. of P was 10.7–5.6% respectively.

Micro assay of nucleic acids. The residues from the extraction with cold chloroform-methanol (see above) were re-extracted with 1 ml. of chloroform-methanol-10 M-HCl (200:100:1, by vol.). The residue was further extracted with 0.5 ml. of acetone-water (9:1, v/v) and with 0.5 ml. of ethanol-ether (3:1, v/v). The residues were freeze-dried and weighed. About 2.5% of the dry residue was used for determination of residue protein (see below). Acid-soluble material was extracted twice with 1.0 ml. of cold (4°) 0.3 M-HClO₄. The residue of DNA and RNA were isolated by centrifugation at 20000g for 10 min. at 4°.

Determination of RNA. A micro adaptation of the method of Hess & Thalheimer (1965) was used. The RNA was extracted with 1 ml. of 1.6 M-HClO₄ at 4° for 16 hr. The supernatant was isolated by centrifugation at 20000g for 20 min. at 4° and the *E*₂₆₀ was read. The recovery of 0.54 µg. of added yeast RNA was 90.1%. In the range 5.70–1.38 µg. of RNA, the analytical error was 0.75–0.92%.

Determination of DNA. The fluorimetric method of Kissane & Robins (1958) was used. A 50 µl. sample of 1 M-diaminobenzoic acid was mixed with the residue after RNA extraction. Then 0.1 ml. of 0.6 M-HClO₄ was added after a coupling period of 45 min. at 60°. The tubes were centrifuged at 20000g for 10 min. A further 1.0 ml. of 0.6 M-HClO₄ was added to 40 µl. of supernatant and the fluorescence read [exciting wavelength 433 nm., obtained from the 436 nm. line of the mercury lamp by means of a Jena 433 nm. interference filter (half-width 9 nm., peak transmission 46% at 436 nm.), emitting wavelength 520 nm. (Jena narrow-bandpass filter, maximal transmission at 520 nm.)]. On the basis of a theoretical P content of 9.43%, the DNA standard employed (Sigma; type I; calf thymus) was considered to be 78.4% pure. The calculated values of µg. of DNA/sample were then multiplied by the factor 1.23. The recovery of 6.48 µg. of DNA was 96.7%. The analytical error was 5.1–2.4% in the range 6.48–25.92 µg. of DNA.

Determination of residue protein. To 80–200 µg. of freeze-dried residue from the chloroform-methanol extraction was added 100 µl. of 0.5 M-NaOH; samples, NaOH blanks and

albumin standards were incubated overnight at 37°. The protein content was determined by the method of Lowry *et al.* (1951) modified as a micromethod (Hess & Lewin, 1965). The albumin standard [Serum-Albumin vom Rind, RHD 04; 88% (w/w) protein; Behringwerke A.-G.] was dissolved in distilled water, and an equal volume of 1 M-NaOH was added. Dilutions were made with 0.5 M-NaOH. The recovery of 7.5 µg. of albumin was 93.0%. With 7.84 µg. of albumin the analytical error was 2.8%.

Statistical evaluation. All values obtained are the results of duplicate or quadruplicate determinations; s.e.m. values are given. The Student's *t* test was used to evaluate the level of significance.

Experimental approach. To elucidate the influence of antibodies and complement on the composition of HeLa cells, the medium of the cultures was changed 18 hr. before the experiment. The old medium was exchanged with 10 ml. of fresh medium containing only 5% of horse serum. Then antibody and complement were added. In Expt. A (controls) 0.1 or 7.0 mg. of IgG from non-immunized rabbits + 0.1 ml. of fresh guinea-pig serum/ml. were added; in Expt. B (antibody + complement), 0.1 or 7.0 mg. of IgG from immunized rabbits + 0.1 ml. of guinea-pig serum/ml.; and in Expt. C (antibody), 0.1 or 7.0 mg. of IgG from immunized rabbits + 0.1 ml. of heat-inactivated guinea-pig serum. Complement addition was always performed as the last step, and the incubation time measured from this point. The composition of HeLa cells was evaluated as a function of time.

RESULTS

Specificity of antibody

The IgG isolated by DEAE-cellulose chromatography of sera from immunized and non-immunized rabbits exhibited only one precipitation line possessing slow γ -globulin mobility in immunoelectrophoresis. This was obtained by diffusion of the IgG fraction against donkey anti-rabbit serum.

The anti-HeLa antibody specificity of the IgG

Table 1. *Time-dependent changes in cell counts, dry weight, protein, DNA and RNA of HeLa cells exposed to IgG*

IgG was from non-immunized rabbits (A) or from rabbits immunized with whole HeLa cell homogenate (B). Both A and B contained active complement. Expt. C corresponds to Expt. B, except for the addition of inactive instead of active complement. Zero-time values were obtained before the addition of antibody and complement. Results are given as means $\pm$ s.d.

Expt.	No. of cultures	Time of incubation (hr.)	$10^{-6} \times$ No. of cells	Dry wt. (mg./culture)	Protein (mg./culture)	DNA (µg./culture)	RNA (µg./culture)
A	8	0	9.1 $\pm$ 0.5	5.4 $\pm$ 0.3	0.81 $\pm$ 0.05	69 $\pm$ 7	147 $\pm$ 24
	16	1	9.6 $\pm$ 1.8	4.7 $\pm$ 0.2	1.19 $\pm$ 0.07	81 $\pm$ 6	136 $\pm$ 11
	16	3	11.5 $\pm$ 1.7	5.2 $\pm$ 0.8	1.33 $\pm$ 0.04	80 $\pm$ 11	159 $\pm$ 30
	8	27	11.9 $\pm$ 2.2	5.8 $\pm$ 0.8		56 $\pm$ 10	166 $\pm$ 12
B	8	0	9.1 $\pm$ 0.5	5.4 $\pm$ 0.3	0.81 $\pm$ 0.05	69 $\pm$ 7	147 $\pm$ 24
	16	1	8.3 $\pm$ 2.2	4.4 $\pm$ 0.2	0.86 $\pm$ 0.03	80 $\pm$ 6	139 $\pm$ 6
	16	3	8.0 $\pm$ 1.4	4.9 $\pm$ 0.8	1.02 $\pm$ 0.14	70 $\pm$ 7	132 $\pm$ 11
	8	27	7.8 $\pm$ 3.9	4.3 $\pm$ 1.1		61 $\pm$ 9	131 $\pm$ 18
C	4	0	5.7 $\pm$ 1.5	5.4 $\pm$ 0.3	2.13 $\pm$ 0.06	71 $\pm$ 6	121 $\pm$ 26
	4	1	6.7 $\pm$ 0.5	6.1 $\pm$ 0.7	2.99 $\pm$ 0.90	71 $\pm$ 8	111 $\pm$ 13
	8	3	6.4 $\pm$ 0.1	6.0 $\pm$ 1.1	1.78 $\pm$ 0.32	68 $\pm$ 1	98 $\pm$ 25

from immunized animals was tested against whole HeLa antigen and against the horse serum employed in the culture medium. Only the HeLa antigen was precipitated with the isolated IgG. Two immunoprecipitates appeared, both of which also developed with the Fab fragment but not with the Fc fragment, thus demonstrating the antibody character of the IgG fraction isolated. Only the crude 400 000g-min. fraction gave rise to immunoprecipitation (two lines) with the IgG fraction.

Biochemical changes induced by IgG and complement

Number of cells, nucleic acids, residue protein and dry weight. In Expts. A and B, changes in all these parameters were found when the HeLa cells were exposed to IgG and guinea-pig serum (Table 1).

The transient increase in the cellular content of DNA after addition of guinea-pig serum may indicate that a number of the cells entered the S phase of the division cycle after this treatment. In fact, the cell counts of control cultures increased within 27 hr. (Table 1).

Time-lapse photography of some of the cells exposed to antibody and complement revealed mitosis, but the cell duplications were unable to compensate for the simultaneous cytolysis (Table 1). These changes were not significant when IgG was added alone (Expt. C). The increase in DNA was followed by an increase in RNA (Table 1, Expt. A) in the control cultures. In cells exposed to antibody and complement, a decrease in RNA was

Table 2. *Kinetic changes in cholesterol and phospholipid and cholesterol/phospholipid molar ratio of cultivated HeLa cells*

Expt. A, IgG from non-immunized rabbits + complement added; Expt. B, anti-HeLa IgG + complement added; Expt. C, anti-HeLa IgG + inactivated complement added. Zero-time values were obtained before addition of antibody and complement. Results are given as means $\pm$ S.D.; S.D. is not indicated where only four cultures are involved.									
Amount of IgG (mg./ml. of medium)			$10^{15} \times$ Cholesterol (moles/cell)						
Expt. and probability	No. of cultures	Time of incubation:							
		0	1hr.	3hr.	5hr.	27hr.			
0.1	A	16	25.8	24.1 $\pm$ 3.1	32.1 $\pm$ 5.3	26.7 $\pm$ 0.3	20.1 $\pm$ 0.8		
	B	16	25.8	33.6 $\pm$ 2.3	43.6 $\pm$ 13.9	40.2 $\pm$ 5.2	29.1 $\pm$ 1.5		
	P (B-A)			< 0.05	< 0.2	< 0.1	< 0.05		
	C	8	17.5 $\pm$ 10.9	20.8 $\pm$ 4.0	23.4 $\pm$ 3.5				
7.0	A	8	21.7 $\pm$ 2.3	29.4 $\pm$ 2.6	29.2				
	B	8	26.3 $\pm$ 1.6	46.1 $\pm$ 4.2	66.1 $\pm$ 6.3				
	P (B-A)		< 0.3	< 0.005	< 0.1				
	C	8	26.3 $\pm$ 1.6	45.9 $\pm$ 8.4	34.8 $\pm$ 4.3				
	P (C-A)		< 0.3	< 0.2	< 0.2				
$10^{15} \times$ Phospholipid (moles/cell)									
0.1	A	16	76.0	61.5 $\pm$ 3.9	83.7 $\pm$ 3.4	70.3 $\pm$ 1.7	67.1 $\pm$ 0.8		
	B	16	76.0	72.2 $\pm$ 11.4	95.8 $\pm$ 3.5	73.6 $\pm$ 5.9	77.8 $\pm$ 1.3		
	P (B-A)			< 0.3	< 0.005	< 0.6	< 0.005		
	C	8	67.0	92.2 $\pm$ 17.4	86.7 $\pm$ 23.4				
	P (C-A)			< 0.5					
7.0	A	8	45.9 $\pm$ 9.6	58.7 $\pm$ 16.3	59.5 $\pm$ 16.3				
	B	8	61.6 $\pm$ 3.8	89.9 $\pm$ 3.3	142.9 $\pm$ 4.6				
	P (B-A)		< 0.2	< 0.1	< 0.02				
	C	8	61.6 $\pm$ 3.8	102.3 $\pm$ 2.6	99.2 $\pm$ 2.1				
	P (C-A)		< 0.2	< 0.05	< 0.1				
Cholesterol/phospholipid molar ratio									
0.1	A	16	0.34 $\pm$ 0.05	0.39 $\pm$ 0.05	0.38 $\pm$ 0.06	0.38 $\pm$ 0.03	0.30 $\pm$ 0.04		
	B	16	0.34 $\pm$ 0.05	0.46 $\pm$ 0.08	0.46 $\pm$ 0.15	0.48 $\pm$ 0.07	0.37 $\pm$ 0.05		
	C	8	0.28 $\pm$ 0.18	0.23 $\pm$ 0.06	0.22 $\pm$ 0.07				
7.0	A	8	0.48 $\pm$ 0.11	0.50 $\pm$ 0.15	0.49 $\pm$ 0.13				
	B	8	0.43 $\pm$ 0.04	0.51 $\pm$ 0.05	0.46 $\pm$ 0.05				
	C	8	0.43 $\pm$ 0.04	0.45 $\pm$ 0.14	0.36 $\pm$ 0.09				
	Medium	4	0.37						

observed (Table 1, Expt. B), associated with the appearance of an increasing amount of RNA in the culture media, in the range 1.0–12 μ g./8ml. of pooled medium.

Cholesterol and phospholipid. The amount of cholesterol and phospholipid per cell increased after addition of IgG. This increase depended on the amount of antibody added and whether active or inactivated guinea-pig serum was added simultaneously. At 1 hr. after exposing cells to 7.0mg. of antibody (IgG) and 0.1 ml. of fresh complement/ml. of medium, cholesterol increased to a value of 175%, and phospholipid to a value 145%, of the initial values. At 3 hr. after the addition of anti-HeLa IgG and complement, cholesterol increased to 250% and phospholipids to 230% of the initial value (Table 2). Anti-HeLa IgG and inactivated complement, or IgG from non-immunized rabbits, induced a transient increase in the cellular content of cholesterol and phospholipid. The maximum was reached within 2–3 hr. after their addition and initial values were attained within 5–6 hr. (Table 2).

Table 2 also illustrates that, in spite of the changes in the cellular content of cholesterol and phospholipid described, the cholesterol/phospholipid ratio per cell remained fairly constant (Expts. A and (C), but increased insignificantly with increasing amounts of antibody (Expt. B).

The changes in the cellular content of cholesterol esters were similar to the changes in total cholesterol, with respect to the order of magnitude as well as to the kinetic changes.

Phospholipid pattern. The fractionated phospholipids of cells exposed to IgG and complement were compared with the phospholipid patterns of cells exposed to specific antibodies in the presence of active or inactivated complement. In all cases an increase of lysophosphatidylcholine occurred. This increase was transient, reaching a maximum 3 hr. after the addition of IgG isolated from immunized animals. The value reached 9–14 moles/100 moles of total phospholipid, depending on the amount of antibody added and whether active or inactivated guinea-pig serum (complement) was added simultaneously (Table 3).

The content of lysophosphatidylcholine in cultures exposed to IgG isolated from non-immunized rabbits was in the range 0.5–4.5 moles/100 moles. A concomitant decrease appeared in the molar percentage of phosphatidylcholine (Table 3).

Tables 4, 5 and 6 summarize the values obtained when the molar percentages of lysophosphatidylcholine and phosphatidylcholine in cultures exposed to IgG isolated from non-immunized rabbits (and complement) are subtracted from the content of these lipids in cultures exposed to specific antibody (and complement). In spite of the relative

Table 3. *Quantitative two-dimensional chromatography of phospholipids in HeLa cells exposed to control IgG (A) or anti-HeLa IgG and complement (B)*

The table summarizes three experiments, i.e. each value represents the average of 12 cultures. Zero-time values refer to pattern before the additions. D gives the composition of the fresh medium.

Expt.	Phospholipid	Time of incubation	...	Composition (moles/100 moles of total phospholipid)		
				0	1hr.	3hr.
A	Lysophosphatidylcholine			1.2	0.0	2.0 $\pm$ 1.3
	Sphingomyelin			6.9 $\pm$ 3.2	10.3 $\pm$ 4.6	10.0 $\pm$ 4.4
	Phosphatidylserine			8.5 $\pm$ 3.1	7.5 $\pm$ 1.8	9.1 $\pm$ 1.3
	Lysophosphatidylethanolamine			5.8 $\pm$ 1.7	3.6 $\pm$ 1.6	5.1 $\pm$ 2.7
	Phosphatidylethanolamine			32.7 $\pm$ 4.6	31.9 $\pm$ 2.7	31.7 $\pm$ 3.1
	Phosphatidylcholine			45.2 $\pm$ 5.6	47.4 $\pm$ 5.6	46.7 $\pm$ 6.6
B	Lysophosphatidylcholine			1.2	5.0 $\pm$ 2.9	10.6 $\pm$ 2.6
	Sphingomyelin			6.9 $\pm$ 3.2	10.9 $\pm$ 6.3	5.4 $\pm$ 1.5
	Phosphatidylserine			8.5 $\pm$ 3.1	6.5 $\pm$ 4.5	8.9 $\pm$ 1.3
	Lysophosphatidylethanolamine			5.8 $\pm$ 1.7	11.0 $\pm$ 2.4	8.6 $\pm$ 6.2
	Phosphatidylethanolamine			32.7 $\pm$ 4.6	30.2 $\pm$ 3.3	28.3 $\pm$ 3.3
	Phosphatidylcholine			45.2 $\pm$ 5.6	38.7 $\pm$ 4.3	37.8 $\pm$ 3.4
D	Lysophosphatidylcholine			1.1 $\pm$ 0.5		
	Sphingomyelin			6.8 $\pm$ 2.4		
	Phosphatidylserine			—		
	Phosphatidylinositol			0.9 $\pm$ —		
	Lysophosphatidylethanolamine			5.5 $\pm$ 2.7		
	Phosphatidylethanolamine			10.6 $\pm$ 0.1		
	Phosphatidylcholine			75.7 $\pm$ 7.2		

Table 4. *Relative molar proportion of lysophosphatidylcholine in control cultures (A) subtracted from antibody-treated (B or C)*

P , probability of $\bar{B}-\bar{A}$ or $\bar{C}-\bar{A}$. $\bar{\sigma}$, estimated standard error of the difference between the arithmetic means, $\bar{A}$, $\bar{B}$, and $\bar{C}$. Expt. A, IgG+active complement added; Expt. B, anti-HeLa IgG+active complement added; Expt. C, anti-HeLa IgG+inactive complement added.

IgG (mg./ml. of medium)	No. of cultures	Time of incubation							
		1hr.		3hr.		5hr.		27hr.	
		$\bar{B}-\bar{A}$ ($\bar{\sigma}_{\bar{B}-\bar{A}}$)	P	$\bar{B}-\bar{A}$ ($\bar{\sigma}_{\bar{B}-\bar{A}}$)	P	$\bar{B}-\bar{A}$ ($\bar{\sigma}_{\bar{B}-\bar{A}}$)	P	$\bar{B}-\bar{A}$ ($\bar{\sigma}_{\bar{B}-\bar{A}}$)	P
0.1	8	0.0 (2.3)		0.7 (1.9)		3.9 (1.1)	<0.05	0.3 (0.4)	
7.0	8	-1.2 (0.7)	<0.2	11.8 (2.8)	<0.01	8.5 (2.3)	<0.05	1.8 (1.3)	<0.2
	8	3.5 (0.6)	<0.005	13.9 (1.2)	<0.001	5.8 (1.1)	<0.005		
	8	9.3 (1.8)	<0.01	6.5 (0.5)	<0.001				
	8	2.3 (0.8)	<0.1	12.0 (0.6)	<0.001				
7.0		$\bar{C}-\bar{A}$ ($\bar{\sigma}_{\bar{C}-\bar{A}}$)	P	$\bar{C}-\bar{A}$ ($\bar{\sigma}_{\bar{C}-\bar{A}}$)	P				
	4	5.9 (2.7)	<0.2	5.3 (5.4)	<0.3				
	4	0.8 (0.8)	<0.5	4.9 (1.0)	<0.05				

Table 5. *Relative molar proportion of phosphatidylcholine in control cultures (A) subtracted from antibody-treated (B or C)*

Symbols and Expts. A, B, and C are as described in Table 4. Each value involved eight cultures.

IgG (mg./ml. of medium)	Time of incubation											
	1hr.			3hr.			5hr.			27hr.		
	$\bar{B}-\bar{A}$ ($\bar{\sigma}_{\bar{B}-\bar{A}}$)	P		$\bar{B}-\bar{A}$ ($\bar{\sigma}_{\bar{B}-\bar{A}}$)	P		$\bar{B}-\bar{A}$ ($\bar{\sigma}_{\bar{B}-\bar{A}}$)	P		$\bar{B}-\bar{A}$ ($\bar{\sigma}_{\bar{B}-\bar{A}}$)	P	
0.1	3.1 (2.0)	<0.2		-3.1 (1.7)	<0.1		0.7 (2.2)	<0.7		1.7 (2.7)	<0.6	
7.0	1.4 (3.6)	<0.7		-7.7 (3.6)	<0.05		-2.7 (3.4)	<0.5		0.5 (2.0)	<0.8	
	-5.1 (2.7)	<0.2		-8.8 (1.8)	<0.005		-2.3 (2.4)	<0.3				
	-0.9 (3.8)	<0.8		-7.1 (3.6)	<0.1							
	-4.4 (5.5)	<0.4		-10.8 (3.1)	<0.005							
7.0	$\bar{C}-\bar{A}$ ($\bar{\sigma}_{\bar{C}-\bar{A}}$)	P		$\bar{C}-\bar{A}$ ($\bar{\sigma}_{\bar{C}-\bar{A}}$)	P							
	-4.1 (3.3)	<0.3		-13.3 (2.7)	<0.05							
	7.3 (3.6)	<0.2		-1.4 (3.8)	<0.7							

Table 6. *Kinetic changes in the content of phosphatidylcholine and lysophosphatidylcholine in cells from HeLa cultures exposed to antibody and complement (B) compared with cells exposed to IgG from non-immunized rabbits and complement (A)*

Results are given as means $\pm$ S.D. Each value involved 16 cultures.

Expt.	Time of incubation ...	$10^{15} \times$ Phosphatidylcholine (moles/cell)		
		1hr.	3hr.	5hr.
		29.9 $\pm$ 1.1	32.5 $\pm$ 2.6	24.9 $\pm$ 0.9
A		27.0 $\pm$ 2.3	32.8 $\pm$ 3.5	27.1 $\pm$ 1.1
B				
Expt.	Time of incubation ...	$10^{15} \times$ Lysophosphatidylcholine (moles/cell)		
		1hr.	3hr.	5hr.
		0.0	1.6 $\pm$ 1.6	3.9 $\pm$ 2.2
A		2.4 $\pm$ 0.3	10.8 $\pm$ 3.5	8.5 $\pm$ 0.9
B				

decrease in the molar percentage of phosphatidylcholine in antibody-exposed cultures compared with control cultures, the absolute cellular content

of phosphatidylcholine was fairly constant, because of the increase of total phospholipid (Table 6).

In cultures exposed to anti-HeLa antibody and

complement, a similar but less significant ($0.8 > P > 0.02$) increase was found in the molar percentage of lysophosphatidylethanolamine (by 1–6 moles/100 moles). The relative content of phosphatidylethanolamine decreased by 1–9 moles/100 moles ($0.8 > P > 0.02$). The content of sphingomyelin decreased slightly (by 2–5 moles/100 moles) in the cultures exposed to antibody and complement ($0.9 > P > 0.2$). The changes in the relative content of phosphatidylserine were variable and insignificant. No significant changes occurred in the ganglioside content (lipid-soluble *N*-acetylneuraminic acid) nor in the lipid-soluble protein (proteolipid).

DISCUSSION

The DNA content of HeLa cells found in the present work is similar to that found by Tedesco & Mellman (1967). However, cultured cells usually have a higher DNA content than cells of mammalian tissues *in situ* (Tsao & Cornatzer, 1967). The proportion of HeLa RNA to DNA agrees with that found by these authors.

The transient increase in DNA per cell occurring after addition of IgG and guinea-pig serum may indicate that a number of the cells entered the S phase of the division cycle after this treatment. In fact, the cell counts of the control cultures increased during the first 28 hr. after addition of guinea-pig serum. Some cells exhibited mitosis, but the cell multiplication was not able to compensate for the immunocytolysis. The increase in cellular RNA after the addition of guinea-pig serum might indicate an increased protein synthesis and was also found by Becker & Levitt (1968).

Addition of anti-HeLa cell antibodies and active complement increased the cellular content of cholesterol, total phospholipids, lysophosphatidylcholine and lysophosphatidylethanolamine, but decreased the molar percentages of phosphatidylcholine and phosphatidylethanolamine during a period of 180 min. However, the cholesterol/phospholipid molar ratio per cell remained constant. These changes were associated with the appearance of juxta-nuclear vacuoles (F. Güttler & J. V. Larsen, unpublished work), but were apparently not specifically related to the presence of complement.

The role of lysophosphatidylcholine in antigen-antibody-complement-mediated immunohaemolysis (Flexner & Noguchi, 1902; Fischer, 1964) has been questioned (Dalmaso & Müller-Eberhard, 1966; Phillips & Middleton, 1965). Smith & Becker (1968) demonstrated a decrease in phosphatidylserine and phosphatidylcholine in sensitized sheep erythrocytes related to the splitting of one fatty acid ester bond/molecule of affected phospholipid. They were, however, unable to identify the product formed by this liberation of free fatty acids. Sheep

erythrocytes contain, however, four times as much sphingomyelin and only 1% of the phosphatidylcholine present in HeLa cells employed in this study (Gier & van Deenen, 1961). Further, erythrocytes cannot form hydrolase-containing vacuoles (Allison, 1966). These findings might explain the fact that the formation of lyso compounds described in the present paper is related to the formation of vacuoles, but probably not to the enzymic activity of complement. This is supported by the demonstration (Dingle, 1965) that the content of lysophosphatidylcholine in lysosomes is higher than in mitochondria.

Recent studies have indicated the presence of lysosomal lipases (Fowler & de Duve, 1967; Mellors & Tappel, 1967; Stoffel & Greten, 1967) including a phospholipase A cleaving phosphatides at the C-2 position (Scherphof, Waite & van Deenen, 1966; Bjørnstad, 1966). The lyso compounds formed are, however, subject to different competitive reactions and will normally not accumulate (van Deenen, 1964, 1965). The transient accumulation of lysophosphatidylcholine found in the present study might thus indicate a temporary imbalance of the diacyl-monoacyl phosphoglyceride cycle (van Deenen, 1965) in cells exposed to antibody. Accumulation of lysophosphatidylcholine might affect the stability of organized membrane structures (Bangham, 1964) and thereby promote fusion of membranes.

Since the molar percentage of lysophosphatidylcholine of the medium was found to be 1.1%, the possibility that the lyso components of the HeLa cells exposed to antibody and complement originate from the medium seems to be excluded.

Berger & Karnovsky (1966) have presented evidence that increased turnover of phosphatides might be relevant to the vesiculation process observed during endocytosis. The constant cholesterol/phospholipid ratio as a function of time after addition of IgG and complement found in the present study might indicate that the increase in the cellular content of lipids is due to an increased synthesis of membrane material. The constant ratio also suggests a control mechanism maintaining the composition of newly formed membranes constant.

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REFERENCES

- Albers, R. W. & Lowry, O. H. (1955). *Analyt. Chem.* **27**, 1829.
- Allison, A. C. (1966). *Proc. Roy. Soc. Med.* **59**, 867.
- Bangham, A. D. (1964). In *Cellular Injury*, p. 167. Ed. by DeReuck, A. V. S. & Knight, J. London: J. and A. Churchill Ltd.

- Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
- Becker, J. & Levitt, J. (1968). *Exp. Cell Res.* **51**, 27.
- Benedetti, E. L. & Emmelot, P. (1968). In *The Membranes*, vol. 4, p. 33. Ed. by Dalton, A. J. & Hagenau, F. New York and London: Academic Press.
- Berger, R. R. & Karnovsky, M. G. (1966). *Fed. Proc.* **25**, 840.
- Bitensky, L. (1963). *Brit. med. Bull.* **19**, 241.
- Bjørnstad, P. (1966). *Biochim. biophys. Acta*, **116**, 500.
- Clausen, J. (1969). In *Laboratory Techniques in Biochemistry and Molecular Biology*, p. 502. Ed. by Work, T. S. & Work, E. Amsterdam: North-Holland Publishing Co.
- Dalmasso, A. P. & Müller-Eberhard, H. J. (1966). *J. Immunol.* **97**, 680.
- Debuch, H. (1964). In *Biochemisches Taschenbuch*, p. 247–251. Ed. by Rauen, H. M. & Kuhn, R. Berlin: Springer-Verlag.
- Dingle, J. T. (1965). Ph.D. Thesis: University of Cambridge.
- Dingle, J. T., Fell, H. B. & Coombs, R. R. A. (1967). *Int. Arch. Allergy, N.Y.*, **31**, 283.
- Dumonde, D. C., Bitensky, L., Cunningham, G. J. & Chayen, J. (1965). *Immunology*, **8**, 25.
- Fahey, J. L. & Horbett, A. P. (1959). *J. biol. Chem.* **234**, 2645.
- Fischer, H. (1964). *Ann. N.Y. Acad. Sci.* **116**, 1063.
- Fiske, C. H. & Subbarow, J. (1925). *J. biol. Chem.* **66**, 375.
- Flexner, S. & Noguchi, H. (1902). *J. exp. Med.* **6**, 277.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
- Fowler, S. D. & de Duve, C. (1967). *J. Cell Biol.* **35**, 41 A.
- Gier, J. & van Deenen, L. L. M. (1961). *Biochim. biophys. Acta*, **49**, 286.
- Glick, D. (1961). *Quantitative Chemical Techniques of Histo- and Cyto-chemistry*, vol. 1, p. 407. New York and London: Interscience Publishers Inc.
- Goldberg, B. & Green, H. (1959). *J. exp. Med.* **109**, 505.
- Green, H., Barrow, P. & Goldberg, B. (1959). *J. exp. Med.* **110**, 699.
- Güttler, F. & Clausen, J. (1969). *Biochem. J.* **114**, 839.
- Heremans, J. F. (1960). *Les Globulines Sériques du Système Gamma*, p. 121. Brussels: Editions Arscia.
- Hess, H. H. & Lewin, E. (1965). *J. Neurochem.* **12**, 205.
- Hess, H. H. & Rolde, E. (1964). *J. biol. Chem.* **239**, 3215.
- Hess, H. H. & Thalheimer, C. (1965). *J. Neurochem.* **12**, 193.
- Kissane, J. M. & Robins, E. (1958). *J. biol. Chem.* **233**, 184.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965). *Immunochemistry*, **2**, 235.
- Marinetti, G. V. (1962). *J. Lipid Res.* **3**, 1.
- Mellors, A. & Tappel, A. L. (1967). *J. Lipid Res.* **8**, 479.
- Paul, J. (1961). *Cell and Tissue Culture*, 2nd ed., p. 88. Edinburgh: E. and D. S. Livingstone Ltd.
- Phillips, B. G. & Middleton, E. (1965). *J. Immunol.* **94**, 40.
- Porter, R. R. (1959). *Biochem. J.* **73**, 119.
- Rhee, K. S. & Dugan, G. R., jun. (1967). *Analyt. Biochem.* **19**, 157.
- Rouser, G., Siaktos, A. N., Fleischer, S. & Simon, G. (1966). In *Biological Membranes*, pp. 5–69. Ed. by Chapman, D. London and New York: Academic Press.
- Scherphof, G. L., Waite, M. & van Deenen, L. L. M. (1966). *Biochem. biophys. Acta*, **125**, 406.
- Skipski, V. P., Peterson, R. F. & Barclay, M. (1964). *Biochem. J.* **90**, 374.
- Smith, J. K. & Becker, E. L. (1968). *J. Immunol.* **100**, 459.
- Stahl, E. (1962). *Dunnschicht-Chromatographie*, p. 5. Berlin: Springer-Verlag.
- Stoffel, W. & Greten, H. (1967). *Hoppe-Seyl. Z.* **348**, 1145.
- Tedesco, T. A. & Mellman, W. J. (1967). *Exp. Cell Res.* **45**, 230.
- Tsao, S.-S. & Cornatzer, W. E. (1967). *Lipids*, **2**, 41.
- van Deenen, L. L. M. (1964). In *Metabolism and Physiological Significance of Lipids*, pp. 155–177. Ed. by Dawson, R. M. C. & Rhodes, R. New York: John Wiley and Sons Inc.
- van Deenen, L. L. M. (1965). In *Progress in the Chemistry of Fats and other Lipids*, vol. 8, part 1. Ed. by Holman, R. T. Oxford: Pergamon Press Ltd.
- Webster, G. R. & Folch, J. (1961). *Biochim. biophys. Acta*, **49**, 399.
- Wieme, R. J. (1959). *Agar Gel Electrophoresis*, pp. 87–122. Brussels: Editions Arscia.
- Williams, J. H. & Witter, R. F. (1965). *J. Lipid Res.* **6**, 461.