THE FUSION OF HEN ERYTHROCYTES BY LIPID-SOLUBLE AND SURFACE-ACTIVE IMMUNOLOGICAL ADJUVANTS

Q. F. AHKONG, J. F. HOWELL, W. TAMPION and J. A. LUCY

Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, 8 Hunter Street, London WC1N 1BP, U.K.

Received 5 March 1974

1. Introduction

A considerable number of chemical agents are now known to induce avian erythrocytes to fuse into multinucleated cells under defined conditions in vitro. These include lysolecithin [1, 2], various unsaturated and saturated fatty acids, their methyl and glyceryl esters, and the fat-soluble vitamins, retinol and α-tocopherol [3], a mixture of polylysine and phospholipase C [4], Ca^{2+} at pH 10.5 [5], and liposomes containing phosphatidylserine [6].

Some of these fusogenic lipids also behave as immunological adjuvants: viz, retinol [7], lysophosphatidylcholine [8], oleic acid [9] and linoleic acid [10]. The mechanism of adjuvant action has not yet been resolved but it is noteworthy that many adjuvants have surface-active properties [9,11] and are able to labilize lysosomal membranes [7,12]. Actions on cell or lysosomal membranes may therefore be some importance for adjuvant activity. In the work reported here, we have investigated the ability of a number of known adjuvants to fuse hen erythrocytes. Our findings indicate that a relationship exists between adjuvant and fusogenic properties, and it seems possible that adjuvant and fusogenic behaviour may both involve the introduction of instability into the structure of cell membranes. A preliminary communication on this work has been published [13].

2. Materials and methods

The test system used for investigating the ability of agents to cause the fusion of hen erythrocytes was that described by Ahkong et al. [3], in which washed hen erythrocytes (3×10^8 cells/ml) were incubated at 37°C with the ultrasonically dispersed test substance in a buffered salt solution (pH 5.6 or 7.4) containing Dextran 60C (80 mg/ml). Samples of the treated cells were examined by phase contrast microscopy at frequent intervals over a period of 4 hr for the formation of multinucleated cells.

The following compounds were investigated: Bayol F (Serva, Micro-Bio Laboratories Ltd., London W. 11, U.K.); Arlacel A and DL-sphingosine sulphate (Sigma, London); dodecylamine and tetradecane (BDH Chemical Co. Ltd., Poole, Dorset, U.K.); Freund's adjuvants (complete and incomplete) (Difco Laboratories, Surrey, U.K.); octadecylamine (K & K Laboratories Inc., Plainview, U.S.A.); Span 20, Span 40, Span 60, Span 80, hexadecane, octadecane, hexadecylamine and octadecenylamine (Koch-Light Laboratories Ltd., Colnbrook, U.K.).

Thin layer chromatography [14] showed that the commercial samples of non-ionic surfactants used were mixtures; Arlacel A and Span 80 were therefore treated with Amberlite IR-120 and IRA-400 ion exchange resins to remove charged components [15]. The treated samples (1.5 g) were introduced onto a column of silicic acid (100 g, Mallinckrodt, 100 mesh; Camlab, Cambridge, U.K.), and the lipids eluted into two major fractions with chloroform. Ester determinations [16] and the R_f values of the purified fractions on tlc plates were consistent with the principle components of these two fractions being mono- and di-ester derivatives (fig. 1).

Electron micrographs of negatively-stained preparations of egg lecithin, before and after treatment with

North-Holland Publishing Company — Amsterdam

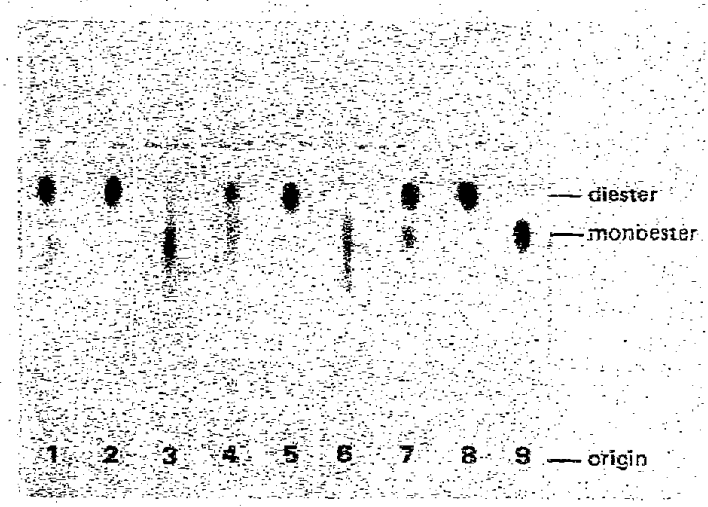

Fig. 1. Separation by thin layer chromatography of purified fractions of non-ionic surfactants. The adsorbent was silica gel H (Merck). Development was in chloroform–acetone (2:1) after which the plate was dried and visualised with iodine vapour [14]. The samples (150 μg) are: 1) unpurified Arlacel A, 2) its diester fraction, 3) its monoester fraction, 4) unpurified Span 80, 5) its diester fraction, 6) its monoester fraction, 7) unpurified ethylene glycol monooleate, 8) its diester fraction, 9) its monoester fraction.

adjuvants and related compounds, were taken at initial magnifications of 50–75 000 × with an A.E.I. EM6b electron microscope.

3. Results and discussion

Commercial preparations of non-ionic surfactants that have been reported to behave as adjuvants, Arlacel A (mannide monooleate) [12, 17], Span 80 (sorbitan monooleate) [11], and Span 20 (sorbitan monolaurate) [9], were tested for their ability to fuse hen erythrocytes. Cell fusion was observed on using sonicates (2 mg/ml) of these substances. Significantly, fusion was not seen with two related long-chain saturated esters tested; Span 40 (sorbitan monopalmitate) and Span 60 (sorbitan monostearate). This is consistent with the previously reported observations that fusion occurs with esters of both medium-chain saturated, and longer chain unsaturated fatty acids, but not in general with esters of palmitic and stearic acids [3].

Span 80 [18] and Arlacel A [19] are mixtures of partial esters of a polyol (sorbitol or mannitol) and its anhydrides. When samples of these materials were separated into mono- and dioleate fractions (see Materials and methods: fig. 1), the monooleate fractions of both surfactants (0.1 mg/ml) induced the fusion of hen erythrocytes (fig. 2). The dioleate fractions were less effective; few binucleate cells being observed after treatment with a higher concentration (0.2 mg/ml). The behaviour of the oleate esters of sorbitol and mannitol therefore parallels that of the mono- and dioleate esters of glycerol [3].

Several cationic lipids that are known to be active as adjuvants [11, 20] were able to cause cell fusion in the test system: DL-sphingosine, and a number of amines with long chains, dodecylamine, hexadecylamine, octadecylamine and octadecenylamine, were fusogenic at a concentration of 0.1 mg/ml. Octadecenylamine (oleylamine) was most effective in causing hen erythrocytes to fuse. This amine has been used successfully to produce homokaryons from mouse fibroblasts [21] and in the present work it was employed to form heterokaryons from mouse fibroblasts and hen erythrocytes.

The widely used Freund's adjuvants, complete and incomplete (0.1 mg/ml) also fused hen erythrocytes: both constituents of the incomplete adjuvant, the mineral oil (Bayol F) and the emulsifier (Arlacel A) were active fusogenic agents. *n*-Hexadecane, which is one of the major components of Bayol F [22], has known adjuvant properties [23], and straight chain hydrocarbons (C_{15-20}) have been used to induce experimental allergic encephalomyelitis in guinea pigs [24]. It is interesting that incubation of hen erythrocytes with tetradecane, hexadecane and octadecane (0.1 mg/ml) also led in our experiments to the formation of multinucleated hen erythrocytes.

Many fusogenic fatty acids and their esters interact with egg lecithin to yield new structures in negatively-stained preparations that differ both from the characteristic structure of lecithin liposomes and from the relatively unstructured appearance of the fusogenic lipid alone [25, 26]. In the present work the addition of the monooleate fractions of Arlacel A or Span 80 to dispersed lecithin resulted in a marked modification of the characteristic liposome structure: occasionally new lamellar and vesicular structures were seen when lecithin was treated with these agents (fig. 3). Ethylene glycol monooleate, which is fusogenic [3], but appears not to have been investigated as an adjuvant, was more

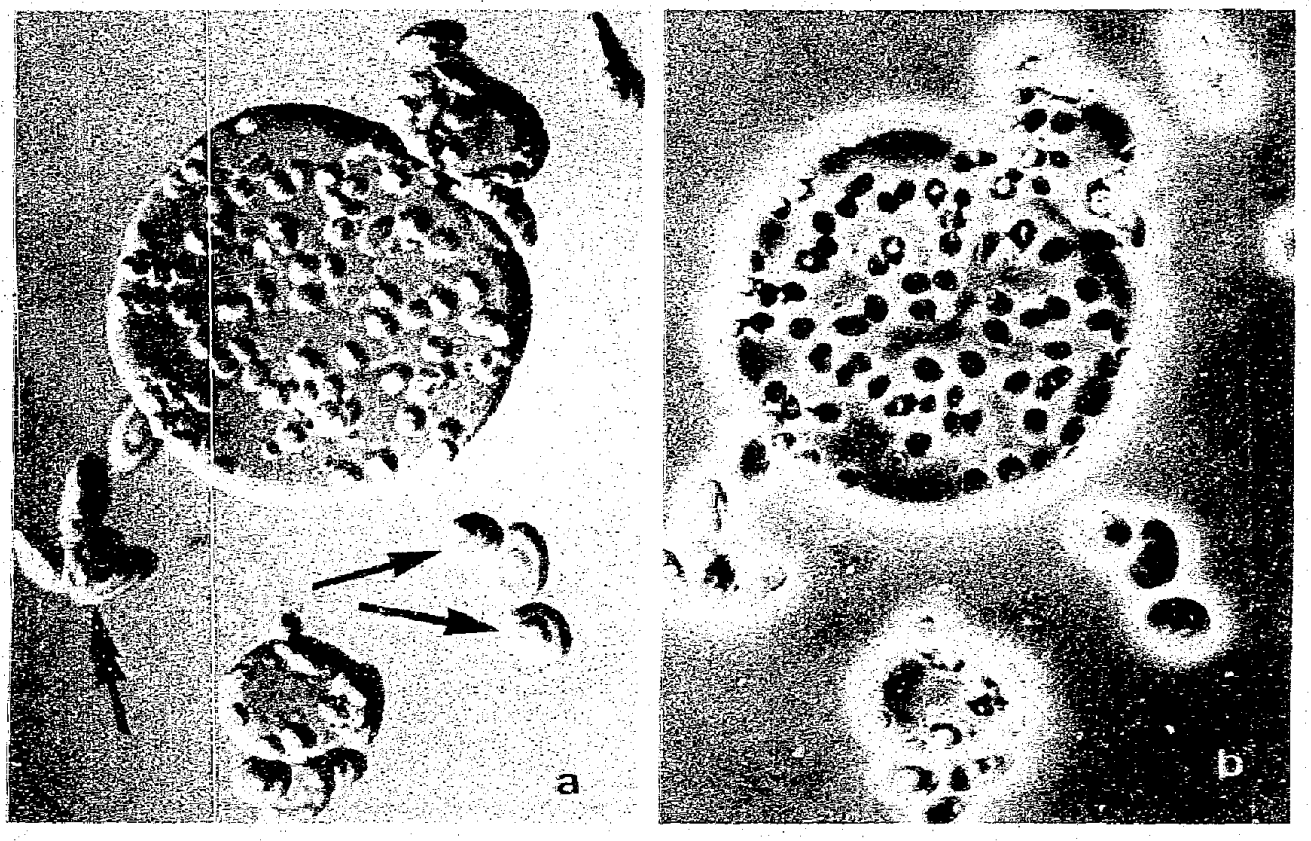

Fig. 2. Light micrographs (X 600) of hen erythrocytes treated with the monoolenate fraction of Arlacel A (0.1 mg/ml) for 45 min at 37°C, pH 5.6. (a) Nomarski differential-interference-contrast microscopy; (b) phase-contrast microscopy. A large cell, in which more than 50 nuclei can be distinguished, and two cells each containing some ten nuclei are seen. Two mononucleate cells (single arrows) that have the swollen appearance known to precede fusion [3], and disc-shaped cells (double arrow) that have been little affected by the added adjuvant are also present.

active in this respect. Dioleate fractions of ethylene glycol, Arlacel A and Span 80 (fig. 1), which were less effective in causing cell fusion, did not interact in this way with lecithin thus behaving like glyceryl dioleate [25, 26].

In summary, a number of non-ionic surface active molecules, long-chain amines, and straight chain hydrocarbons, all of which have adjuvant properties have been found to be fusogenic towards hen erythrocytes. Some of these molecules have also been shown to interact physically with aqueous dispersions of egg lecithin. It is suggested that adjuvant activity and fusogenic properties may both be expressions of the ability of these molecules to interact with biological membranes. Cell fusion might itself play a role in adjuvant function should transfer of material through cytoplasmic connections between macrophages and lymphocytes [27–29], and between lymphocytes and target cells [30], prove to be of functional immunological importance. Alternatively, if thymus-derived lymphocytes produce soluble mediators that affect B cells or macrophages [31], adjuvants may facilitate secretion of mediators by affecting the fusion of membranes that occurs in exocytosis.

Acknowledgements

This work was supported by the Medical Research Council; the electron microscope was provided by the Science Research Council.

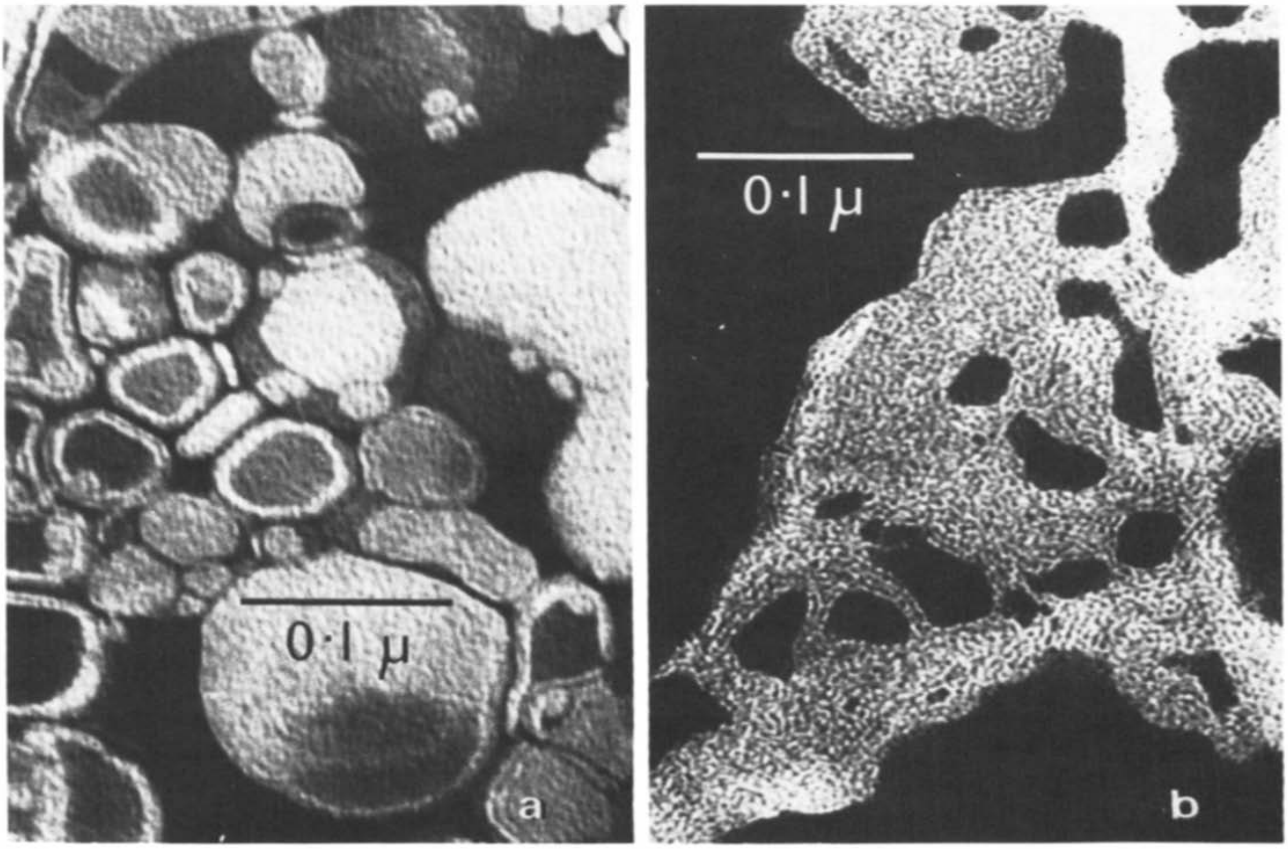

Fig. 3. Negatively-stained electron micrographs (× 230 000) of (a) a sonicated aqueous dispersion of egg lecithin (1.25 mg/ml) in 2% potassium phosphotungstate, pH 5.6, brought to 37°C before electron microscopy, (b) aqueous, sonicated lecithin (1.25 mg/ml) mixed at 37°C with a sonicated dispersion of the monooleate fraction of Span 80 (1.25 mg/ml), prepared for electron microscopy as in (a). Electron microscopy was undertaken as described previously [26].

References

[1] Howell, J. I. and Lucy, J. A. (1969) FEBS Letters 4, 147–150.
[2] Poole, A. R., Howell, J. I. and Lucy, J. A. (1970) Nature 227, 810–813.
[3] Ahkong, Q. F., Fisher, D., Tampion, W. and Lucy, J. A. (1973) Biochem. J. 136, 147–155.
[4] DeBoer, E. and Loyter, A. (1971) FEBS Letters 15, 325–327.
[5] Toister, Z. and Loyter, A. (1971) Biochim. Biophys. Acta 241, 719–724.
[6] Papahadjopoulos, D., Poste, G. and Schaeffer, B. E. (1973) Biochim. Biophys. Acta 323, 23–42.
[7] Dresser, D. W. (1968) Nature 217, 527–529.
[8] Westphall, O., Fischer, H. and Munder, P. G. (1970) Proc. 8th Int. Congr. Biochem. 319–320.
[9] Dresser, D. W. (1961) Nature 191, 1169–1171.
[10] Dresser, D. W. (1968) Clin. Expll. Immunol. 3, 877–888.
[11] Gall, D. (1966) Immunology 11, 369–386.
[12] Spitznagel, J. K. and Allison, A. C. (1970) J. Immunol. 104, 119–127.
[13] Ahkong, Q. F., Cramp, F. C., Fisher, D., Howell, J. I., Tampion, W. and Lucy, J. A. (1972) Biochem. J. 130, 44–45P.
[14] Berlin, B. S. and Wyman, R. (1971) Proc. Soc. Expll. Biol. Med. 136, 1363–1368.
[15] Ahkong, Q. F. (1973) M. Sc. Thesis. University of Manchester Institute of Technology.
[16] Snyder, F. and Stephens, N. (1959) Biochim. Biophys. Acta 34, 244–245.
[17] Berlin, B. S. (1963) Annl. Allergy 21, 82–90.
[18] Sahasrabudhe, M. R. and Chadha, R. K. (1969) J. Am. Oil. Chem. Soc. 46, 8–12.
[19] O'Neill, H. J., Yamauchi, T. N., Cohen, P. and Hardegree, M. C. (1972) J. Pharm. Sci. 61, 863–867.
[20] Younger, J. S. and Axelrod, V. (1963) J. Immunol. 90, 1–11.

[21] Bruckdorfer, K. R., Cramp, F. C., Goodall, A. H., Verrinder, M. and Lucy, J. A. J. Cell Sci. In press.
[22] O'Neill, H. J., Yamauchi, T. N., Cohen, P. and Hardegree, M. C. (1972) J. Pharm. Sci. 61, 1292–1296.
[23] Hoyt, A., Knowles, R. G., Moore, F. J. and Smith, C. R. (1957) Am. Rev. Tuberc. 75, 624–629.
[24] Shaw, C. M., Alvord, Jr., E. C. and Kies, M. W. (1964) J. Immunol. 92, 24–27.
[25] Ahkong, Q. F., Cramp, F. C., Fisher, D., Howell, J. I., Tampion, W., Verrinder, M. and Lucy, J. A. (1973) Nature New Biol. 242, 215–217.
[26] Howell, J. I., Fisher, D., Goodall, A. H., Verrinder, M. and Lucy, J. A. (1973) Biochim. Biophys. Acta 332, 1–10.
[27] Schoenberg, M. D., Mumaw, V. R., Moore, R. D. and Weisberger, A. S. (1964) Science 143, 964–965.
[28] Clarke, J. A., Salsbury, A. J. and Willoughby, D. A. (1971) J. Pathol. 104, 115–118.
[29] Yokomuro, K. and Nozima, T. (1972) J. reticuloendothelial Soc. 11, 579–598.
[30] Seelin, D., Wallach, D. F. H. and Fischer, H. (1971) Eur. J. Immunol. 1, 453–458.
[31] Maillard, J. and Bloom, B. R. (1972) J. Exptl. Med. 136, 185–190.