Heterogeneity of Macrophage Cytophilic Antibodies in Immunized Mice

Serum antibodies cytophilic for macrophages are produced by guinea-pigs, rabbits and mice. They are detectable in vitro by virtue of their ability to attach to macrophages from normal animals, conferring on them an affinity for antigen after free serum has been removed from the medium. They are of particular interest because of their possible association with delayed-type hypersensitivity[1]. Recently an investigation has been made in this laboratory of the factors determining the production of macrophage cytophilic antibodies and the development of delayed-type hypersensitivity to sheep erythrocytes in mice[2]. The production and titre of cytophilic antibodies in mice parallel the development of delayed-type hypersensitivity as revealed by footpad testing.

In this communication we report differences in the properties of macrophage cytophilic antibodies appearing in serum 7 days after immunization with sheep erythrocytes, in association with delayed-type hypersensitivity ('early serum'), and those reappearing in serum 28 days after a booster injection ('hyperimmune serum').

Non-inbred Swiss mice were injected s.c. with 0.2 ml of an emulsion of equal volumes of 10% sheep erythrocytes in saline and Freund's complete adjuvant. One group was bled 7 days after immunization to give a pool of early serum. Other mice from the same group were footpad tested with sheep erythrocytes and showed pure delayed reactions. The second group was reinjected i.p. with 0.2 ml of 5% sheep erythrocytes in saline 21 days after immunization; 7 days later some were bled to give a pool of hyperimmune serum, while others were footpad tested and showed combined Arthus (4 h) and delayed reactions. The techniques used are described in detail elsewhere[2]. Macrophage cytophilic antibodies were titrated by the addition of dilutions of serum to monolayers of cultured mouse peritoneal macrophages, washing the cultures to remove free serum, adding sheep erythrocytes, washing again and recording the attachment of erythrocytes to macrophages as 0 to ++++. Complement-fixing antibodies were titrated by a Microtiter adaptation of immune adherence, and direct hemagglutinating antibodies in a Microtiter system. In preliminary experiments it was found that the macrophage cytophilic antibody activity in early sera was lost on repeated freezing and thawing (4 cycles), whereas that in hyperimmune sera was unaffected by such treatment. For this reason pooled sera were stored in aliquots and frozen and thawed only once before use.

In attempts to elucidate the nature of the cellular receptors to which cytophilic antibodies become attached, several workers have treated cells with proteolytic enzymes such as trypsin and papain. In the case of guinea pig macrophages and cytophilic antibodies such treatment has been found not to abolish but actually to increase the subsequent uptake of cytophilic antibodies[3]. Monolayers of mouse macrophages were treated with trypsin (Difco Trypsin 1:250, 1 mg/ml) at 37 °C for 30 min and thoroughly washed before being used for the titration of macrophage cytophilic antibodies in early or hyperimmune serum. Control cultures were incubated in Hanks's solution without trypsin. The results of the titrations are shown in the Table. Trypsin treatment virtually abolished the capacity of macrophages to take up cytophilic antibodies from early serum, but increased their capacity to take up cytophilic antibodies from hyperimmune serum (as reflected by the subsequent uptake of sheep erythrocytes). This finding strongly suggested that different receptors exist for the early and hyperimmune cytophilic antibodies. In other experiments it was found that trypsinization of macrophages after incubation with serum removed the cytophilic factor taken up from early serum but not that taken up from hyperimmune serum. The effects of trypsin have proved to be reproducible with several other pools of early and hyperimmune serum.

The possibility that early cytophilic antibodies might be 19 S and hyperimmune antibodies 7S immunoglobulins was tested by examining the effect of 2-mercaptoethanol on the activity in the 2 types of serum. Both were resistant to 2-ME. Electrophoretic separation of the 2 types of cytophilic antibody was attempted using Pevikon blocks[4]. The fractions were eluted from 1 or 2 cm cuts and protein determinations were made by the Folin method. The protein was precipitated by dialysis against saturated ammonium sulphate in the cold. The centrifuged precipitates were dissolved by dialysis against saline followed by Hanks's solution. The fractions were tested for cytophilic antibody activity (with normal and trypsinized macrophages), for complement-fixing antibodies and for hemagglutinating antibodies. Aliquots were also examined by immunoelectrophoresis and starch gel electrophoresis[5].

[1] D. S. NELSON and S. V. BOYDEN, Br. med. Bull. *23*, 15 (1967).
[2] D. S. NELSON and P. MILDENHALL, Aust. J. exp. Biol. med. Sci. (in press), 1967.
[3] J. G. HOWARD and B. BENACERRAF, Br. J. exp. Path. *47*, 193 (1966); E. GOWLAND, personal communication; S. KOSSARD and D. S. NELSON, in preparation.
[4] H. J. MÜLLER-EBERHARD, Scand. J. clin. Lab. Invest. *12*, 33 (1960). The conditions for the present experiments were: barbitone buffer, 0.12 *M*, pH 8.6, electrophoresis for 22 h at 5 °C, gradient 5 V/cm.
[5] O. SMITHIES, Biochem. *61*, 629 (1955).

Effect of trypsin treatment of mouse macrophages on uptake of cytophilic antibodies

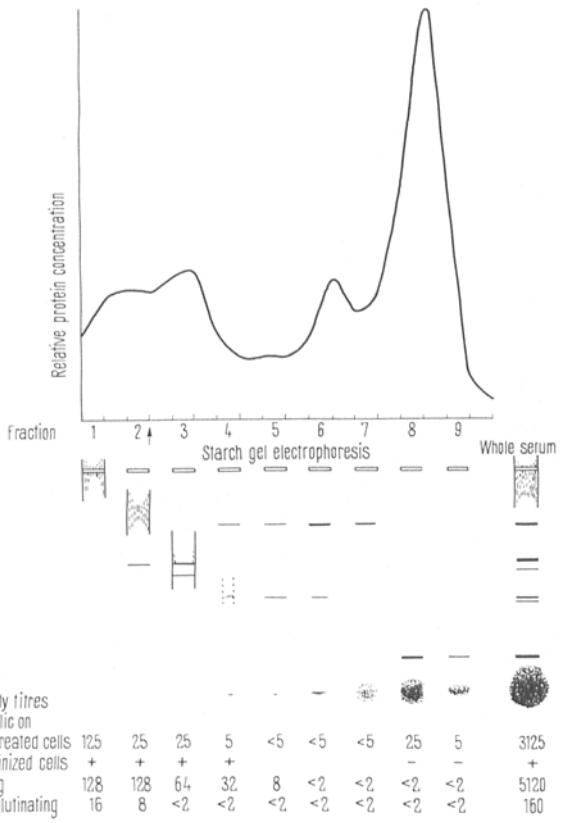
Anti-sheep erythrocyte serum	Cells	Dilution of serum					
		5	25	125	625	3125	No serum
Early[a]	Normal	+	++	+	±[c]	±[c]	−
	Trypsinized	±[c]	−	−	−	−	−
Hyperimmune[b]	Normal	++++	+++	+++	++	+	−
	Trypsinized	++++	++++	++++	+++	++	−

[a] Mice bled 7 days after s.c. injection of sheep erythrocytes in adjuvant. [b] Mice bled 28 days after s.c. injection of sheep erythrocytes in adjuvant and 7 days after i.p. injection of sheep erythrocytes in saline. [c] ± reactions are equivocal and are not considered significant.

In hyperimmune serum cytophilic, complement-fixing and hemagglutinating activities were found in fractions containing γ- and β-globulins. Cytophilic, but not other antibody activity, was also found in the fraction containing albumin with some α_1-globulin, the latter being detected on starch gel electrophoresis but not on immunoelectrophoresis using rabbit anti-mouse serum. The cytophilic activity in the γ-β area was greater when the fractions were titrated on trypsinized macrophages, whereas that in the albumin-α_1 fraction was not detectable with trypsinized macrophages (Figure). Fractionation of the early serum revealed cytophilic activity only in the albumin-α_1-globulin fractions; this activity was not detectable when trypsinized macrophages were used. Early serum contained complement-fixing antibodies to very low titre (1:10) and this activity was lost on fractionation. Electrophoresis of normal serum yielded fractions with similar components but no cytophilic activity.

It is tempting to speculate that the 'albumin-α_1' cytophilic factor attaching to a trypsin sensitive receptor may be implicated in delayed-type hypersensitivity in the mouse, especially as there is a correlation between the titre of 'early' antibody and the intensity of delayed footpad reactions in primarily immunized animals[2]. The finding of cytophilic activity in such a fraction is also reminiscent of an unconfirmed report by RAUCH and FAVOUR[6] that delayed-type hypersensitivity in the guinea-pig was transferable with large amounts of a Cohn fraction (IV-1) containing mainly α-globulin. The relationship of this cytophilic factor to conventional immunoglobulins also requires investigation: does it represent a new class of immunoglobulin having only cytophilic activity, or does it represent isolated chains or 3.5 S fragments[7] of conventional immunoglobulins? Some caution is required in the use of the term 'antibody' to describe a factor which is not a conventional immunoglobulin. The specificities both of the stimuli required to induce its formation and of the affinities of the factor, once formed, for foreign material, are at present under investigation. To date, only one of 7 pools of sera from mice injected with adjuvant alone has been found to confer on macrophages an affinity for sheep erythrocytes. Macrophages treated with early anti-sheep erythrocyte serum have shown an affinity for sheep erythrocytes, but not human, guinea pig or other mouse erythrocytes.

Experiments are in progress to characterize further the nature, significance and origin of the cytophilic factor in early serum. The present experiments, together with those of other workers, indicate a very considerable heterogeneity among cytophilic antibodies in general. This is now apparent with respect to: (a) the type of cell to which cytophilic antibodies can attach, e.g. macrophage cytophilic antibodies appear to be distinct from spleen cell cytophilic antibodies[1], which may in fact attach to lymphocytes[8]; (b) the serum fractions within which cytophilic antibodies are found, e.g. among IgM (19 S) and IgG (7 S) in rabbits and mice[9] as well as in the albumin-α_1 fraction described here; (c) the receptors to which different classes of macrophage cytophilic antibodies attach[10].



Antibody titres										
Cytophilic on										
untreated cells	125	25	25	5	<5	<5	<5	25	5	3125
trypsinized cells	+	+	+	+				−	−	+
C' fixing	128	128	64	32	8	<2	<2	<2	<2	5120
Hemagglutinating	16	8	<2	<2	<2	<2	<2	<2	<2	160

Pevikon C-870 block electrophoresis of a pool of hyperimmune serum, showing relative protein concentration (above), starch gel electrophoretic patterns of fractions and results of antibody titrations of fractions and of whole serum. Mouse serum pool – 28 days, sheep erythrocytes in adjuvant s.c. plus boost at 21 days. The effects of trypsin treatment of macrophages on the uptake of cytophilic antibodies (as reflected by the subsequent uptake of sheep erythrocytes) are indicated as: + = increased uptake; − = abolition of uptake. Similar patterns have been observed with other pools of hyperimmune serum.

Résumé. Des souris immunisés avec des globules rouges de mouton dans l'adjuvant de Freund produisent deux types de facteurs cytophiles pour macrophages. L'un s'attache aux macrophages trypsinisées et se trouve parmi les immunoglobulines. L'autre ne s'attache pas aux macrophages trypsinisées et se rencontre dans une fraction de sérum contenant de l'albumine et de la globuline-α_1.

D. S. NELSON, S. KOSSARD and PRUDENCE E. COX

Department of Bacteriology, University of Sydney (N.S.W., Australia), 9th January 1967.

[6] H. C. RAUCH and C. B. FAVOUR, Ann. N.Y. Acad. Sci. *87*, 231 (1960).
[7] O. KUWAHARA, S. SHINKA, M. IMAMISHI, B. MIYAGAWA, T. MORI and T. AMANO, Biken's J. *9*, 1 (1966).
[8] H. KOPROWSKI and M. V. FERNANDEZ, J. exp. Med. *116*, 467 (1962); S. KOSSARD and D. S. NELSON, in preparation.
[9] W. E. PARISH, Nature *208*, 594 (965); E. SORKIN, personal communication.
[10] Supported by grants from the Australian Research Grants Committee, the National Health and Medical Research Council of Australia and the Postgraduate Medical Foundation, University of Sydney.