Location of Nucleotide Pyrophosphatase and Alkaline Phosphodiesterase Activities on the Lymphocyte Surface Membrane

By ERIKA R. ABNEY, W. HOWARD EVANS* and R. MICHAEL E. PARKHOUSE
National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

(Received 5 May 1976)

1. Isolated mouse spleen lymphocytes hydrolysed UDP-galactose added to the medium. Nucleotide pyrophosphatase activity that accounted for this hydrolysis was enriched to a similar extent as alkaline phosphodiesterase and 5'-nucleotidase in a lymphocyte plasma-membrane fraction. 2. The cell surfaces of mouse spleen and thymus lymphocytes were iodinated with 125I by using the lactoperoxidase-catalysis method. Detergent extracts of the cells were mixed with a purified anti-(mouse liver plasma-membrane nucleotide pyrophosphatase) antiserum and the immunoprecipitates analysed by polyacrylamide-gel electrophoresis. Only one major radioactive component, similar in size (apparent mol.wt. 110000–130000) to the liver enzyme, was observed. 3. Electrophoresis of an iodinated spleen plasma-membrane fraction indicated peaks of radioactivity, including one of apparent mol.wt. 110000–130000. 4. When detergent extracts of spleen lymphocytes were passed through a Sepharose-bead column containing covalently attached anti-(nucleotide pyrophosphatase) antiserum, the nucleotide pyrophosphatase activity was retained by the beads, whereas protein and leucine naphthylamidase activity were eluted. 5. The results indicate that nucleotide pyrophosphatase and alkaline phosphodiesterase activities are due to the location of the same or similar enzymes at the outer aspect of the lymphocyte plasma membrane. Some possible functions of enzymes at this location are discussed.

Plasma-membrane enzymes whose active sites face the external medium rather than the cytoplasm have been designated 'ectoenzymes' (De Pierre & Karnovsky, 1974a). It now appears that a number of mammalian plasma-membrane marker enzymes can be classified as ectoenzymes (De Pierre & Karnovsky, 1974b; Trams & Lauter, 1974) and that these enzymes, when located in different tissues and organs may possess similar properties (Riemer & Widnell, 1975). Nucleotide pyrophosphatase was shown to be an ectoenzyme in hepatocytes (Evans, 1974; Bischoff et al., 1976), and in various cultured cell lines (Sela et al., 1972; Deppert et al., 1974). The present studies show that the enzyme activity present in lymphocyte plasma membranes occupies a similar position.

Experimental

Animals

Specific pathogen-free mice 8–12 weeks old of the inbred strain CBA/Ca (equivalent to CBA/H) were used as a source of normal spleen and thymus lymphocytes. Mice were deprived of thymus-derived lymphocytes (T-lymphocytes) by being thymectomized as young adults and subsequently irradiated and reconstituted with syngeneic foetal liver (Miller & Mitchell, 1969). The lymph nodes and spleens of these mice [(CBA×C57) F1 hybrids] had 70 and 80% immunoglobulin-bearing cells (B-lymphocytes) respectively. Also, spleen lymphocytes from these mice contained less than 5% of cells with the antigenic determinant Thy-1 on their surface; this antigen has been shown to be a marker for T-lymphocytes (Reif & Allen, 1964). A lymphocyte plasma-membrane fraction was prepared from male mice, 6 months old, of the CBA/Ca strain.

Preparation of antiserum

Nucleotide pyrophosphatase was purified from a sarcosyl extract of mouse liver plasma membranes by rate-zonal centrifugation in sucrose/sarcosyl gradients, followed by gel filtration (Evans et al., 1973). Polyacrylamide-gel electrophoresis and chemical analyses indicated that the enzyme was a sialoglycopeptidol of apparent mol.wt. 120000–130000. Antiserum, raised in rabbits against the purified enzyme suspended in Freund's complete adjuvant (Difco Labs., Detroit, MI, U.S.A.), was purified as a y-globulin fraction by (NH4)2SO4 precipitation and gel filtration on Sephadex G-200 columns (120cm×2.5cm) (Gurd et al., 1972). Antiserum was concentrated by Diaflo filtration on UM 50 filters (Amicon, High Wycombe, Bucks., U.K.) to 8 mg/ml and immunoelectrophoretic analyses
indicated one component. The antiserum was covalently coupled to CNBr-activated Sepharose 4B as recommended by the manufacturers (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

**Preparation of lymphocyte cell suspensions**

Lymphocytes were teased from minced mouse spleens into a phosphate-buffered saline medium (124 mM-NaCl / 4 mM-KCl / 2 mM-KH2PO4 / 8 mM-Na2HPO4), pH 7.4, and were separated from erythrocytes and damaged lymphocytes by layering on to a cushion of Isopaque/Ficoll solution [14% (w/v) Ficoll (Pharmacia)/32.8% (w/v) metrizoate (Isopaque) solution (Nyegaard and Co. A.S., Oslo, Norway) (12:5, v/v)] and centrifuging with rapid acceleration in plastic tubes at 20°C for 15 min at 2000g (Davidson & Parish, 1975). The lymphocytes present at the interface were collected, and resuspended in phosphate-buffered saline, pH 7.4, containing 10 mM-glucose. They were then divided into two equal portions, one of which was homogenized in a small tight-fitting homogenizer, and the breakdown of UDP-galactose determined immediately as described below.

**Preparation of plasma membranes**

A plasma-membrane fraction was prepared from washed spleen lymphocytes of 70 mice. Spleen cell suspensions were prepared by mincing the organs in a Moulinex parsley mincer with Hanks gelatin (Dresser & Greaves, 1973), that was adjusted to pH 7.4 by addition of NaHCO3. Cells were washed twice in Hanks gelatin and once in 10 mM-Tris/0.14M-NaCl, adjusted to pH 7.4 by addition of HCl. Cells (approx. 7.4 x 10^9) were ruptured by a single passage through a Stansted disruption press (Stansted Fluid Power, Bentfield Road, Stansted, Essex, U.K.) operating at a disrupting valve pressure of 138 kPa (Crumpton & Snary, 1974). A low-speed pellet was centrifuged by collecting the cell homogenate at 5000g for 15 min. An intermediate-speed pellet was collected by centrifuging the supernatant at 31000g for 30 min. This pellet was resuspended in 10 mM-Tris/HCl, pH 7.4, and re-centrifuged at 31000g for 30 min before resuspension in 10 ml of 37% (w/v) sucrose/10 mM-Tris/HCl, pH 7.4, and overlayed with 25% (w/v) sucrose/10 mM-Tris/HCl, pH 7.4. After centrifuging overnight (18 h) at 97000g (Beckman SW27 rotor) a plasma-membrane fraction was collected at the 25–37% sucrose interface; the pellet at the bottom consisted mainly of endoplasmic reticular membranes (Crumpton & Snary, 1974).

125I labelling of membrane proteins

Lymphocyte cell suspensions were labelled with 125I by using lactoperoxidase (Marchaloni et al., 1971), washed once in ice-cold phosphate-buffered saline (130 mM-NaCl/4 mM-KCl/10 mM-Na2HPO4, pH 7.4) and then lysed for 10 min at 4°C in 1% Nonidet P40 (Shell Chemicals, London W.C.2, U.K.) in phosphate-buffered saline containing 1 mM-phenylmethylsulphonyl fluoride and 10 mM-iodoacetamide (re-crystallized; added to limit proteolysis). The lysate, after centrifuging at 30000g for 15 min, was passed over Sephadex G-25 equilibrated in the solution used for lysis of the cells. Uptake of 125I by the cells was 30–40% of added radioactivity, and at least 95% of the trichloroacetic acid-precipitable radioactivity was recovered in the supernatant after centrifugation of the cell lysate. Radioactivity determined in the excluded peak from the G-25 column was 70–90% trichloroacetic acid-precipitable and accounted for 10–15% of the total radioactivity applied. A ‘non-specific’ immunological precipitate was first formed in the radioactive sample by addition of normal rabbit serum (10 μl) and a goat anti-(rabbit immunoglobulin G) serum (100 μl). The resulting precipitate was removed by centrifugation, and then further precipitation was effected by addition of rabbit anti-(mouse plasma-membrane nucleotide pyrophosphatase) antiserum (120 μg) and, after 30 min, goat anti-(rabbit immunoglobulin G) serum (100 μl). The immunological precipitations were performed at 4°C. Specific precipitates were washed three times with ice-cold 0.5% Nonidet P40 in phosphate-buffered saline (see above for composition), once with 50 mM-sodium phosphate, pH 7, and then dissolved by heating at 100°C for 10 min in 50 mM-Na2HPO4/NaH2PO4, pH 7.0, containing 2% (w/v) sodium dodecyl sulphate. When the samples were reduced, the solvent also included 1 mM-dithiothreitol and iodoacetamide was added (final concn. 100 mM) after the heating step; for non-reduced samples iodoacetamide was present at 100 mM. Internal 131I-labelled markers (Abney & Parkhouse, 1974) were added to the samples, which were then resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Summers et al., 1965). After electrophoresis the gels were sliced into 1 mm segments and radioactivity was determined by using a gamma counter (Packard model 5230).

Lymphocyte plasma membranes washed in 0.15 M-NaCl/5 mM-Tris/HCl buffer, pH 7.4, were iodinated by using lactoperoxidase, and a glucose/glucose oxidase enzymic system was used to generate H2O2 (Hubbard & Cohn, 1973; Evans, 1974). Approx. 1 mg of lymphocyte plasma membranes was iodinated with 0.5 mCi of 125I in 0.5 ml of phosphate-buffered saline/10 mM-glucose, containing 65 μg of lactoperoxidase (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) and 100 μl of glucose oxidase (from Aspergillus niger; approx. 100 units; Sigma). The membranes were washed several times in phosphate-buffered saline before dissolution
in 1% sodium dodecyl sulphate/5mm-mercaptoethanol/10mm-Tris/HCl, pH 7.4, by heating at 90°C for 5 min for polyacrylamide-gel electrophoresis (Evans, 1974).

**Enzymic determinations**

5'-Nucleotidase, alkaline phosphodiesterase, leucine naphthalamidase activities and protein were determined as previously described (Evans et al., 1973).

Nucleotide pyrophosphate was assayed with UDP-galactose as substrate as described by Sela et al. (1972) and Mookerjea & Yung (1975). Enzyme activities of disrupted lymphocytes and plasma membranes are determined at pH 8.6, since maximum hydrolysis of sugar nucleotides occurred at alkaline pH (Sela et al., 1972; Evans et al., 1973). However, comparison of the enzyme activities of intact and disrupted lymphocytes was made at pH 7.6 when cells remain viable and about 50% of the activity is measured (Sela et al., 1972). The complete incubation mixture contained in 100 μl: 10–80 μg of protein; 3 μmol of cacodylate buffer, pH 8.6, or 5 μmol of KH₂PO₄/Na₂HPO₄, pH 7.6; 2nmol of UDP-galactose (10⁴ c.p.m.); 0.5 μmol of MnCl₂; 5 μl of a 10% solution of Triton X-100 (used in pH 8.6 incubations only). Enzymic activity was measured at 37°C for 15 min and stopped by adding 100 μl of ethanol and 50 μl of acetic acid. Particulate material was removed by centrifuging the reaction mixture at 3000rev./min for 5 min, and applying a portion of the supernatant to Whatman 3MM paper. Reaction products were separated by descending chromatography for 18 h in a solvent system containing ethanol/1M-sodium acetate buffer, pH 3.8 (15:6, v/v), and monitored by scanning in a Packard radiochromatogram scanner model 7201 before cutting out strips corresponding to the peak areas for determination of radioactivity by scintillation counting in a toluene-based scintillant [4g of 2,5-diphenyloxazole and 0.1g of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene dissolved in 1 l litre of toluene]. By using the appropriate standards, it was found the UDP-galactose was hydrolysed initially to galactose 1-phosphate and then to galactose, the latter reaction occurring especially with isolated plasma membranes.

**Results**

Although 5'-nucleotidase is a well established lymphocyte plasma-membrane marker (Crumpton & Snary, 1974), there is little information available on the subcellular location of nucleotide pyrophosphatase and alkaline phosphodiesterase activities. Table 1 shows that all enzymes were enriched in a lymphocyte plasma-membrane fraction, relative to the cell homogenate. The hydrolysis of UDP-galactose to UMP and galactose 1-phosphate by nucleotide pyrophosphatase is the first of a two-step reaction that proceeds further, catalysed by a monoester phosphohydrolysis activity, to yield free sugar and nucleosides. This two-stage enzymic hydrolysis of sugar nucleotides has been examined in detail by Sela et al. (1972) and Deppert et al. (1974) in cultured cell lines. Further details of the range of substrates hydrolysed by the nucleotide pyrophosphatase of lymphocyte plasma membranes have yet to be established, but a similar general specificity towards the pyrophosphate bands of NAD, ATP etc. as in hepatocytes (Evans et al., 1973) is to be expected.

The most direct method of demonstrating a location on the external face of the plasma membrane of an enzymic activity is to show that substrates impermeable to cells are rapidly hydrolysed when

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>5'-Nucleotidase (μmol/h per mg of protein)</th>
<th>Alkaline phosphodiesterase (μmol/h per mg of protein)</th>
<th>UDP-galactose pyrophosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Galactose 1-phosphate and galactose produced (c.p.m./h/µg of protein)</td>
</tr>
<tr>
<td>Intact cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell homogenate</td>
<td>240</td>
<td>0.90 (1)</td>
<td>0.35 (1)</td>
<td>6800 (1)</td>
</tr>
<tr>
<td>75000g-min pellet</td>
<td>200</td>
<td>0.31 (0.97)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90000g-min pellet</td>
<td>5</td>
<td>3.4 (9.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90000g-min supernatant</td>
<td>11</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>2</td>
<td>16.3 (18.1)</td>
<td>7.3 (20.7)</td>
<td>102700 (15.1)</td>
</tr>
<tr>
<td>Endoplasmic- reticulum membranes</td>
<td>3</td>
<td>1.6 (4.6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Distribution of lymphocyte-membrane enzymes

The enzymic activities of spleen lymphocytes, cell homogenates and subcellular fractions were determined. The preparation details and the incubation conditions are described in the Experimental section. Values in parentheses indicate specific activity relative to that of cell homogenate.

Vol. 159
Fig. 1. Characterization of lymphocyte surface proteins immunoprecipitated after addition of a anti-(mouse liver plasma-membrane nucleotide pyrophosphatase) antiserum

(a) Normal spleen lymphocytes, (b) lymphocytes from spleens from eight mice, (c) and (d) thymus cells. Lymphocytes were isolated and labelled with $^{125}$I as described in the Experimental section, and specific immunoprecipitates were either unreduced (a–c) or reduced and alkylated (d), and then resolved in 7% (w/v) polyacrylamide gels (----) that also contained the following reduced and alkylated internal $^{125}$I-labelled markers (-----): immunoglobulin Ms (IgMs) ($\mu_2\lambda_2$), $\mu$-chains ($\mu$), $\gamma$-chains ($\gamma$) and light chains (L). The anti-(mouse liver plasma-membrane nucleotide pyrophosphatase) antiserum immunoprecipitated 0.32, 0.26 and 0.52% of the total $^{125}$I radioactivity precipitated by addition of trichloroacetic acid to lysates of labelled normal spleen lymphocyte, spleen lymphocytes from B mice and thymocytes respectively.
added to the medium. Table 1 indicates that washed spleen lymphocytes and disrupted lymphocytes hydrolysed UDP-galactose. However, De Pierre & Karnovsky (1974a) and Trams & Lauter (1974) have shown that this approach to demonstrate ectoenzymic activity on cell surfaces is fraught with technical difficulties and can yield equivocal results. In the present case, the difference between a pH optimum of 8.5–9.0 of nucleotide pyrophosphatase/alkaline phosphodiesterase activities (Evans et al., 1973; Sela et al., 1972) and a pH of 7.4–7.6 necessary to maintain lymphocyte viability and hence the permeability of the cell membrane towards added substrates, compounds these difficulties. To circumvent such problems, advantage was taken of combining techniques for enzymically iodinating cell-surface components with the availability of an antisera raised against the purified mouse hepatocyte enzyme to investigate whether the enzyme, if iodinated on the lymphocyte surface membrane, could be immunoprecipitated. In addition, this technique yields information on the molecular size of the enzyme.

Lactoperoxidase-catalysed iodination of lymphocytes (Vitetta & Uhr, 1973) and erythrocytes (Hubbard & Cohn, 1973) results mainly in the labelling of tyrosine residues exposed to the medium. The nature of the labelled molecules on the lymphocyte surface membrane was studied directly by extraction of 125I-labelled cells with the detergent Nonidet P40, which, in the present experiments, solubilized >90% of the radioactivity present in washed lymphocytes. Addition of a mouse anti-(liver plasma-membrane nucleotide pyrophosphatase) antiserum to detergent extracts of lymphocytes resulted in an immunoprecipitate, that, when analysed by polyacrylamide-gel electrophoresis, contained only one major peak of radioactivity of apparent mol.wt. 110000–115000 in non-reducing conditions (Figs. 1a–c) and 130000 under reducing conditions (Fig. 1d). The iodinated protein was present on both B and T classes of lymphocytes (see the Experimental section). The extremely close correspondence of the molecular weight of the iodinated immunoprecipitated product to that of the mouse liver plasma-membrane enzyme strongly suggested that nucleotide pyrophosphatase was iodinated, and thus located at the outer aspect of the lymphocyte plasma membrane.

Iodination of isolated spleen lymphocyte plasma membranes showed that one of the peaks of radioactivity present was of apparent mol.wt. 130000 (Fig. 2), in agreement with other reports (Ladoulis et al., 1974; Juliano & Behar-Bannelier, 1975; Anderson et al., 1976). A component of similar apparent molecular weight to the immunoprecipitated peak is thus available for iodination in isolated plasma membranes. Since isolated lymphocyte plasma membranes consist mainly of closed vesicles of a right-side out configuration (Allan & Crumpton, 1970; Misra et al., 1974), this result can also be interpreted to suggest a location for nucleotide pyrophosphatase on the outer aspect of the plasma membrane.

---

**Table 2. Retention of alkaline phosphodiesterase activity on a Sepharose 4B column containing covalently bound liver anti-(plasma-membrane nucleotide pyrophosphatase) antiserum**

<table>
<thead>
<tr>
<th>Addition to column (mg)</th>
<th>Phosphodiesterase activity (µmol/h)</th>
<th>Leucine naphthylamidase activity (µmol/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery from column</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery (%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2. Polyacrylamide-gel electrophoresis 125I-radioactivity profile of enzymically iodinated lymphocyte plasma membranes**

The plasma-membrane fraction was prepared and labelled with 125I by using the lactoperoxidase/glucose oxidase catalysis method described in the Experimental section. The apparent-molecular-weight scale was obtained by using a series of reoviral protein markers of known molecular weight.
To demonstrate a direct combination of the mouse liver plasma-membrane nucleotide pyrophosphatase antiserum with the enzyme in lymphocytes attempts were made to demonstrate antigen–antibody interaction. Passage of Nonidet P40 extracts of spleen lymphocytes through a Sepharose column containing covalently attached antiserum against mouse liver plasma-membrane nucleotide pyrophosphatase indicated retention of enzyme activity, whereas protein and leucine naphthylamidase activity were recovered (Table 2). This result suggested a direct combination between the liver anti-(nucleotide pyrophosphatase) antiserum and the enzyme of lymphocytes.

Discussion

Enzymically catalysed iodination of perfused liver and isolated hepatocytes showed that nucleotide pyrophosphatase is a sialoglycoprotein of apparent mol. wt. 130000 located at the outer aspect of the rat and mouse hepatocyte plasma membrane (Evans et al., 1973; Evans, 1974; Bischoff et al., 1975, 1976). Previous observations (e.g. Bischoff et al., 1970) showing that various nucleotides were rapidly hydrolysed when added to the isolated haemoglobin-free perfused liver were thus explained. The purified nucleotide pyrophosphatase of mouse liver (Evans et al., 1973) and rat liver (Bischoff et al., 1974) were shown to hydrolyse a range of substrates containing a nucleotide pyrophosphate bond, and also to behave as an alkaline phosphodiesterase, hydrolysing the artificial substrate thymidine 5'-monophosphate p-nitrophenyl ester. The predictions of Touster et al. (1970), based on similarities in the kinetics of hydrolysis of the physiological and artificial substrates by the liver plasma-membrane enzyme, were thus confirmed. However, although more direct evidence for activity towards nucleotide pyrophosphate and phosphodiester bonds in the case of lymphocytes must await the purification of the enzyme, the results strongly argue that the present enzyme has a similar location and substrate specificity in lymphocytes. Isolated lymphocytes hydrolysed UDP-galactose, and this enzyme was found to be increased in specific activity (relative to the homogenate) in a plasma-membrane fraction to an extent similar to that shown by alkaline phosphodiesterase and 5'-nucleotidase activities. An antisemur raised against the purified mouse hepatocyte plasma-membrane enzyme immunoprecipitated a single iodinated component that was of similar molecular size in extracts of lymphocytes that were previously surface-labelled with $^{125}$I. By studying the inhibitory properties of an antisemur raised against mouse liver plasma membranes towards the 5'-nucleotidase activity of mouse and rat liver and pig lymphocyte plasma membranes, Gurd & Evans (1974) showed that there are strong antigenic similarities between plasma-membrane ectoenzymes that cross not only tissue barriers, but also species barriers. Riemer & Widnell (1975) showed that a 5'-nucleotidase partially purified from a number of rat tissues showed similar properties. The present results then add to the picture that there are classes of glycoproteins with enzymic activity on mammalian cell surfaces that show common antigenic and molecular properties. Indeed, studies of cells of disparate function, e.g. lymphocytes and platelets (Tanner et al., 1974), seem also to indicate that polypeptides of similar molecular-weight classes are iodinated by the lactoperoxidase procedure.

The present demonstration of nucleotide pyrophosphatase/alkaline phosphodiesterase activity on the outer aspect of the lymphocyte plasma membrane adds to the list of cells in which this activity has been similarly located, e.g. hepatocytes (Bischoff et al., 1970) and a variety of cultured cell lines (Deppert et al., 1974; Sela et al., 1972). Monneron (1974) also showed histochemically that UDP-galactose was hydrolysed by thymocyte plasma membranes. Since glycosyltransferases are also claimed to be present on the lymphocyte cell surface (Cacan et al., 1976), the presence of two categories of ectoenzymes that may compete for the same substrate should be borne in mind in devising models [see Shur & Roth (1975) for a review] invoking cell-surface glycosyltransferases to explain cell recognition and adhesion. Ectoenzymes hydrolysing ATP were shown to be present on a variety of eukaryotic cell surfaces by Trams & Lauter (1974) and De Pierre & Karnovsky (1974a,b), and nucleotide pyrophosphatase activity may account for at least part of this hydrolytic activity.

The exact function of a nucleotide pyrophosphatase residing on lymphocyte and other mammalian cell surfaces is not fully understood. The enzyme may function to exclude the entry of nucleotides into cells e.g. RNA-degradation products, or may serve to conserve cellular nucleotides, by ensuring, in conjunction with nucleotide phosphohydrolases (e.g. 5'-nucleotidase), their hydrolysis in the plasma membrane to nucleosides. Specific mechanisms exist for the transport of nucleosides into cells (Berlin & Oliver, 1975). Liberation of adenosine into serum by lymphocytes could cause vasodilation (Dobson et al., 1971), thus ensuring movement of these cells through narrow blood vessels. Clearly, knowledge of the topography of plasma-membrane enzymes is a first step towards determining their possible implication in the transport and metabolism of nucleotides by mammalian cells.

E. R. A. thanks the Consejo Nacional de Ciencia y Tecnología de Mexico, Mexico, for financial support.
References

Reif, A. E. & Allen, J. M. V. (1964) J. Exp. Med. 120, 413–433