Metabolic inhibition of plaque-forming cells: comparison of human rheumatoid-factor-producing cells with mouse anti-sheep erythrocyte-producing cells

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Summary While rheumatoid-factor-producing haemolytic plaque-forming cells (RF–PFC) of the human peripheral blood were easily inhibited by cycloheximide, mouse spleen cells immune to sheep red cells (anti-SRC PFC) were inhibited only after prolonged preincubation in the drug. The RF–PFC were easily inhibited by propranolol, while the anti-SRC PFC were not at all inhibited. Vinblastine inhibited both systems equally. These differences are taken to suggest that the RF–PFC have very little preformed antibody in them and therefore depend upon active protein synthesis for their demonstration. In contrast, anti-SRC PFC, which may be predominantly mature plasma cells, generally need no new protein synthesis for their demonstration because of increased quantities of preformed antibody. A possible mechanism is that RF–PFC may represent primarily RF-specific B cells, the RF of which is released by surface immunoglobulin shedding and therefore susceptible to membrane stabilising agents such as propranolol.

We have recently reported on drugs affecting the production and release of an autoantibody, rheumatoid factor (RF), from human peripheral blood lymphocytes (Vaughan et al., 1976). The RF is detected in a plaque-forming cell (PFC) assay. RF–PFC are apparently very sensitive to only slightly adverse conditions in vitro. For instance, maximal numbers of RF–PFC are found only when full tissue culture medium is used in the agarose phase of the assay, rather than the simpler buffers that are generally used in other PFC assays (Vaughan et al., 1976). We observed that RF–PFC can be inhibited easily by agents interfering with protein synthesis, cycloheximide, or puromycin by a microtubule disrupting agent, vinblastine, and by a membrane stabilising agent, D-propranolol (Moore et al., 1978).

We were interested in whether these characteristics of the RF–PFC system were different from what we would find in another, more familiar PFC assay, the anti-sheep red cell (SRC) PFC. Jerne (1974) has shown in the anti-SRC system that antibody secretion is maintained at full rate at 37°C but is stopped by lowering the temperature. Secretion of antibody lags about 30 minutes behind synthesis. He also showed that incubation of the spleen cell suspension at 37°C in the presence of cycloheximide and puromycin at 10 μg per ml reduced the number of plaques. Although synthesis of antibody was probably completely inhibited, the cells continued to secrete antibody for some hours, which was antibody synthesised before exposure of the inhibitors of protein synthesis. Plaque formation in agar was thus mainly due to active secretion of antibody from viable cells.

We have therefore re-examined mouse spleen cells making a primary anti-SRC response to SRC injected intravenously 5 days previously by looking at the effects of cycloheximide and puromycin and also the effects of vinblastine and propranolol. Our results in the anti-SRC system show distinct differences from those found in the RF–PFC system in relation to cycloheximide and propranolol, and we offer some interpretations for the differences noted.

Materials and Methods

 Animals. Balb/c mice 2-3 months old were obtained from Strong Laboratories.

 Spleen cell preparations. The mice were immunised
intravenously with $5 \times 10^8$ prewashed SRC in 0.1 ml normal saline and killed 5 days later by cervical dislocation. Spleens were teased apart at room temperature by forceps and suspended in 10 ml of balanced salt solution (BSS). The suspension was transferred to a 10 ml plastic tube and allowed to sit for 5 minutes for debris to settle. The suspended cells were transferred to another tube and centrifuged for 10 minutes at 1200 rpm at 4°C. The cell pellet was resuspended in BSS, counted, and then adjusted to a final concentration of $5 \times 10^6$ cells per ml in BSS. In some experiments full tissue culture media rather than BSS were used for this suspension, but with no consequent difference in number of PFC found.

**Haemolytic plaque assay.** 0.5 ml of 0.5% agarose (Agarose A-37 Indubiose) at 44°C was mixed with 0.05 ml of 6.7% suspension of SRC and 0.05 ml of the cell suspension which had been preincubated for 30 minutes at room temperature with the inhibitor at the appropriate concentration or normal saline as control. The mixture was poured on to precoated microscope slides, the slides were incubated for 60 minutes at 37°C in a moist chamber, and then immersed in a 1:10 dilution of guinea-pig complement (Pel-Freeze). After an additional 60 minutes at 37°C PFC were enumerated under magnification. Three replicate determinations were made at each concentration.

**Rheumatoid factor PFC assay.** Peripheral blood lymphocytes were obtained from selected patients with rheumatoid arthritis. These patients had clinically active, seropositive arthritis and had been shown to have high numbers of RF-producing cells in their peripheral blood. The lymphocytes were plated for RF-producing cells as described (Vaughan et al., 1976). In brief, the cells were separated by a Ficoll-Hypaque gradient, suspended in fetal calf serum, and $1-5 \times 10^6$ cells were mixed with sensitised sheep RBC in agarose at 44°C. The mixture was poured immediately on to glass slides as above, but incubation was at 37°C for 90 minutes, followed by an additional 90 minutes at 37°C in 1:10 dilution of guinea-pig complement. Sensitisation of the sheep cells for this assay was with rabbit IgG anti-sheep red cell antibody reduced and alkylated so that it could not by itself activate complement, but would still react with rheumatoid factor (Tanimoto et al., 1975).

**Metabolic inhibitors.** Puromycin and cycloheximide were obtained from Calbiochem; vinblastine was from Lilly; α-propranolol was from Ayerst.

In performing preincubation studies with drugs we noted a considerable loss of number of PFC in our controls during the preincubations. The loss was dependent on both time and temperature (Fig. 1). The results were not changed appreciably by carrying out the preincubation in full tissue culture media. In the studies to be reported appropriate controls were carried out to accommodate the decreasing PFC numbers.

Tests for viability of cells exposed to drugs were made with trypan blue dye. Before inhibitor preincubation their viability was at least 90%. In all cases after incubation at least 80% viability was revealed at time of reading. None of the drugs used in these studies had anticomplementary effects in quantitative haemolytic assay of guinea-pig complement against sensitised sheep erythrocytes.

### Results

Cycloheximide easily suppressed RF-PFC in human peripheral blood lymphocytes when added to the agarose phase of the assay (Table 1). This suppression did not occur, however, with anti-SRC PFC in immune spleen cells. Only when the inhibitor was preincubated with the spleen cells at 37°C for 90 minutes and was also included in the agarose was suppression clearly seen (Fig. 2).

**Table 1** Inhibition of rheumatoid-factor plaque-forming cells by drugs incorporated into the agarose

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>PFC % of control</th>
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<tr>
<td>Cycloheximide</td>
<td>50 μg/ml</td>
<td>40±15</td>
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<tr>
<td></td>
<td>10 μg/ml</td>
<td>32±12</td>
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<td></td>
<td>10-4 M</td>
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<td>10-5 M</td>
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<td>5 × 10-5 M</td>
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<td></td>
<td>2.5 × 10-5 M</td>
<td>100±20</td>
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<tr>
<td>Vinblastine</td>
<td>10-4 M</td>
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<td>36±18</td>
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Fig. 2 Effects of cycloheximide on anti-SRC PFC. The inhibitor was preincubated with immune mouse spleen cells and also incorporated in final agarose phase of the assay. The figures in the columns indicate concentrations of cycloheximide (µg/ml).

Propranolol, which at 10⁻⁴ M and 5 x 10⁻⁵ M showed significant suppression of RF-PFC when only in the agarose (Table 1), showed no inhibition of anti-SRC PFC. Preincubation of the drug with the spleen cells for 90 minutes did not change this (Fig. 3).

Fig. 3 Lack of effect of propranolol on anti-SRC PFC. The propranolol was present during preincubation for 90 minutes at 37°C and also in the agarose phase of the assay. The numbers above the bars are concentrations of propranolol (x 10⁻⁵ M).

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Fig. 4 Effects of vinblastine on anti-SRC PFC. The preincubations were for 90 minutes at 37°C. The figures above the columns represent concentrations of vinblastine (M).

Vinblastine, which had suppressed RF-PFC almost completely when added at 10⁻⁴ M to the agarose phase of the assay (Table 1) also suppressed the anti-SRC PFC (Fig. 4). The relative degrees of suppression in the 2 antibody systems seemed comparable. Reversibility of the vinblastine effect was evident when vinblastine was preincubated with the lymphocytes but left out of the agar phase, a manoeuvre which resulted in a final concentration of the vinblastine in the agarose which was 10-fold less than it had been during the preincubation phase of the experiment (Fig. 4).

Discussion

RF-PFC and anti-SRC PFC differed in their behaviour in cycloheximide and propranolol but not in vinblastine. Vinblastine reduced equally the expression of RF-PFC and anti-SRC PFC. Vinblastine disrupts cytoplasmic microtubules, and presumably this is the mechanism for its inhibiting the PFC in both systems examined.

Cycloheximide was clearly inhibitory of RF-PFC when it was added simply to the agarose phase of the assay. It inhibited the anti-SRC PFC, however, only after prolonged preincubation with the cells. We interpret this to mean that most of the antibody-producing cells in the immune mouse spleen, at least as initially isolated, have large stores of preformed antibody in them, which are easily released during the agarose phase of the assay. These PFC do not need continued synthesis to make enough antibody for the formation of haemolytic plaques and therefore are not suppressible with inhibitors of protein synthesis. When the spleen cells are preincubated under conditions favouring loss of such cytoplasmic stores of antibody, the remaining PFC increasingly represent cells in which continued new synthesis of antibody is needed for plaque formation. Under
these conditions suppression of anti-SRC PFC by cycloheximide is easily demonstrated. RF-PFC, being easily suppressed by cycloheximide without preincubation, would therefore appear to be cells with little in the way of preformed stores of antibody. Propranolol suppressed RF-PFC but did not suppress the mouse spleen anti-SRC PFC even with the preincubation. We have supposed that propranolol exerts its effect on RF-PFC by way of its membrane-stabilising effects (Nickerson et al., 1975). Both the D and L forms of propranolol are active in suppression of RF-PFC (Moore et al., 1978). Only the L form has the familiar beta adrenergic blocking effect (Nickerson and Collier, 1975), while both D and L forms have membrane effects. Both prevent capping of membrane immunoglobulin by anti-Fab (Dunne et al., 1978) as is typical for other membrane-stabilising agents (Ryan et al., 1974). Capping is a means by which B cells can shed their membrane-bound Ig into the environment, and we have wondered whether this is the principal means for RF release from RF-PFC of the peripheral blood. RF-PFC might thereby be much more susceptible to inhibition by membrane-stabilising agents than are splenic plasma cells with their endoplasmic reticulums loaded with antibody ready for easy export.

Obviously the differences we have documented may be related simply to whether the antibody-forming cells are taken from the blood or from the spleen. Or they may conceivably represent species differences. In our previous study (Moore et al., 1978), however, we observed that anti-SRC made by human peripheral blood lymphocytes also were not inhibited by propranolol. Species differences therefore are probably not determinant of the propranolol effects. Finally, we may be observing drug differences related to the antibody, being to an exogenous antigen in one instance and an autoantigen in the other.

Of the drugs tested only propranolol has any conceivable use therapeutically. Neither cycloheximide nor vinblastine can be tolerated in the doses needed. In other studies (Moore et al., 1978; Dunne et al., 1978) we have shown propranolol to be immunosuppressive in vitro in concentrations as low as $2 \times 10^{-5}$ M. However, conventional doses of propranolol give peak plasma levels of only $10^{-6}$ M. Possibly massive doses of propranolol, such as have sometimes been given to patients with hypertension (Holland et al., 1976), could provide effective immunotherapy in RA. Alternatively, congeners of propranolol may be found which would be effective at lower doses.

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References


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