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BRIEF COMMUNICATION

An Improved Assay for Haemolytic Plaque-Forming Cells

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Summary. A haemolytic plaque-forming cell assay is described in which the erythrocyte indicator system is a pre-formed monolayer firmly bound to a surface containing charged groups. Certain experimental procedures for which previously available methods were ill-suited are easily performed by this technique. Applications of the assay are discussed.

INTRODUCTION

The haemolytic plaque-forming cell assay (Jerne and Nordin, 1963; Jerne, Nordin and Henry, 1963) has greatly facilitated studies involving antibody-producing cells or their precursors, but has certain limitations resulting from the presence of the gel supporting medium. The permanent preparations obtained are not well suited for morphological studies, since the gel tends to distort cells on drying and may interfere with staining procedures. In attempted radioautography of plaque-forming cells with a low energy isotope such as tritium, even a very thin layer of dried gel may block an unacceptable proportion of radiation, while slight variations in the thickness of the gel can lead to considerable variation in the apparent degree of isotope incorporation. An additional limitation becomes apparent if it is necessary to assay for plaque-forming cells in the presence of large numbers of autologous erythrocytes. For example, the plaques formed by the antibody-producing cells circulating in the blood may be completely masked by the presence of accompanying erythrocytes, and even spleen cell suspensions contain sufficient erythrocytes to cause problems if the cells must be assayed at high concentration.

The haemolytic plaque-forming cell assays introduced by Ingraham (1963) and Ingraham, Biegel, Watanabe and Todd (1967) provide alternative techniques which are useful for certain specialized applications. In addition they provide fairly good conditions for direct examination of living antibody-forming cells by light microscopy, but permanent preparations are not possible and masking of plaques by autologous cells can cause severe problems. Nucleated cells can also obscure plaques if preparations cannot be stained, since under low magnifications unstained nucleated cells may look much like erythrocytes. The monolayer assay (Cunningham, 1965; Cunningham, Smith and Mercer, 1966; Cunningham and Szenberg, 1968) has similar shortcomings, although it is sensitive, rapid, and highly suitable for studies involving either micro-manipulation procedures or direct examination of living cells by light microscopy.

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The assay technique described below has the sensitivity and simplicity of the Cunningham monolayer assay and is just as convenient for micromanipulation procedures or phase contrast examination of living cells *in situ*. However, it has two additional features: it can produce permanent records which are suitable for radioautographic or morphological studies, and it is capable of detecting plaque-forming cells in the presence of large numbers of autologous erythrocytes or inert nucleated cells.

METHODS AND RESULTS

Formation of monolayer of target erythrocytes. The negatively charged erythrocytes are bound to a negatively charged surface by means of the multiple positive charges of a monomolecular film of poly-L-lysine. The glass of microscope slides does not have a strong enough negative charge, but sulphonation of polystyrene sheets or petri dishes by treatment with concentrated sulphuric acid at room temperature for 30 minutes provides an adequately charged surface. Falcon polystyrene tissue culture dishes are similarly modified during manufacture, and are therefore convenient although relatively expensive. The following description is based on the use of Falcon 60 × 15 mm tissue culture petri dishes.

A stock solution of 1 mg/ml poly-L-lysine (MW 30,000–50,000) in phosphate buffered saline (PBS) showed no detectable loss of activity when kept for several months in a polystyrene tube at 4°. For use, it was diluted to 0·025 mg/ml in PBS or phosphate buffer at pH 7·2, and 2 ml added to each 60 × 15 mm petri dish. After 15 minutes at room temperature, the dishes were thoroughly rinsed in PBS or phosphate buffer, and 2 ml of 1 per cent freshly washed sheep erythrocytes (SRBC) in PBS were added immediately. Thorough washing of the SRBC is important since soluble proteins may interfere with the binding reaction. The SRBC were allowed to settle at room temperature for 15 minutes; then gently agitated and allowed to settle again for another 15 minutes. Finally, they were gently agitated again, the resulting suspension was poured off, and any residual free erythrocytes were washed away by rinsing the petri dish in a beaker of PBS. The monolayer of SRBC which remained was kept under PBS until used. It could be stored overnight at 4°, if required, although under such conditions erythrocytes from older stocks tended to lyse or come loose.

Assay for plaque-forming cells. The PBS covering the monolayer was poured off and 2 ml of a suitable dilution of the spleen cell suspension being tested plus 0·2 ml of fresh frozen guinea-pig serum previously adsorbed with SRBC was added. If indirect plaques were to be assayed, appropriate antiserum was added as well. The dishes were left undisturbed at 37° for 45 minutes, during which time the cells settled on to the monolayer and released antibody. Experiments to determine the optimum time for settling and incubation showed that the plaques did not increase in number after 25 minutes, and reached maximum size in 35 minutes. Allowing the cells to settle on to the monolayer at 4° before beginning incubation did not increase the plaque count. Incubation for longer than 2 hours led to micro-plaques, apparently resulting from cell breakdown, since no nucleated cells could be seen in the centre of these plaques. PBS, Eagle's medium and medium 199 (Grand Island Biological) were found to be equally satisfactory for incubation. When this assay method and that of Cunningham *et al.* (1965, 1966, 1968) were compared for sensitivity, no difference was found except when there were only very few plaque-forming cells present in the spleens being assayed. Under such conditions, it was necessary to use minimally diluted spleen cell suspensions. When the Cunningham assay was used the large

number of inert autologous cells present in such suspensions effectively masked the smaller plaques and even made identification of some of the larger plaques rather difficult; however, when the monolayer of target erythrocytes was firmly bound to a petri dish, rinsing the petri dish in a beaker of PBS removed obscuring spleen cells to unmask clearly visible plaques. In reconstruction experiments in which spleen cells from unimmunized mice were used to dilute spleen cells from mice at the height of the immune response, the presence of even 5×10^7 inert nucleated spleen cells per ml did not reduce the plaque count if the spleen cells were rinsed from the monolayer before the plaques were counted; without rinsing, most of the plaques were completely obscured at this cell concentration. Such a rinsing procedure even made it possible to detect plaque-forming cells in a washed but otherwise unmodified preparation of peripheral blood cells. As a check on the sensitivity of the assay under such conditions, reconstruction experiments were carried out using mixtures of washed mouse erythrocytes and spleen cells from immunized mice. The results of one such experiment are summarized in Table 1. Monolayers of SRBC were

TABLE 1

VISUALIZATION AND ACCURATE ENUMERATION OF PLAQUES PREVIOUSLY OBSCURED BY HIGH CONCENTRATIONS OF OVERLYING ISOLOGOUS ERYTHROCYTES

Concentration of added isologous erythrocytes (cells/ml)	Average plaque count (± SD) per 10^5 spleen cells after overlying cells were rinsed from the monolayers
0	91 (±15)
2×10^7	93 (±6)
2×10^8	94 (±12)
2×10^9	99 (±10)

prepared, and 2 ml of mixtures containing various concentrations of washed mouse erythrocytes, 1×10^5 spleen cells from mice immunized with SRBC 4 days previously, and 10 per cent guinea-pig serum adsorbed with SRBC were flooded over the monolayers and incubated for 45 minutes. The loose cells were then removed by inverting the petri dishes under PBS and allowing them to stay in this position for 10 minutes before examination. Ten petri dishes were used for each erythrocyte concentration. It can be seen that the plaque counts obtained do not differ significantly from each other although before rinsing almost all plaques were obscured in those petri dishes which had received the highest concentration of isologous erythrocytes.

Preparation for radioautographic and morphological studies. Since the cells being assayed are merely resting on the monolayer, the central cells of the plaques are readily displaced by incautious handling. Consequently, it is best to fix the central cells in position before the petri dishes are disturbed in any way. We incubated the dishes in a large container (such as a desiccator) which had an air-tight lid; when the plaques appeared, a 50 per cent glutaraldehyde solution was poured into the bottom of this container or soaked into a sponge fastened to the lid. After several hours of exposure to the vapour, the preparations could be gently rinsed in water without loss of cells. If the dishes were not disturbed during incubation and fixation, there was a nucleated cell firmly bound in the centre of almost every plaque. The preparation could then be stained or prepared for radioautography.

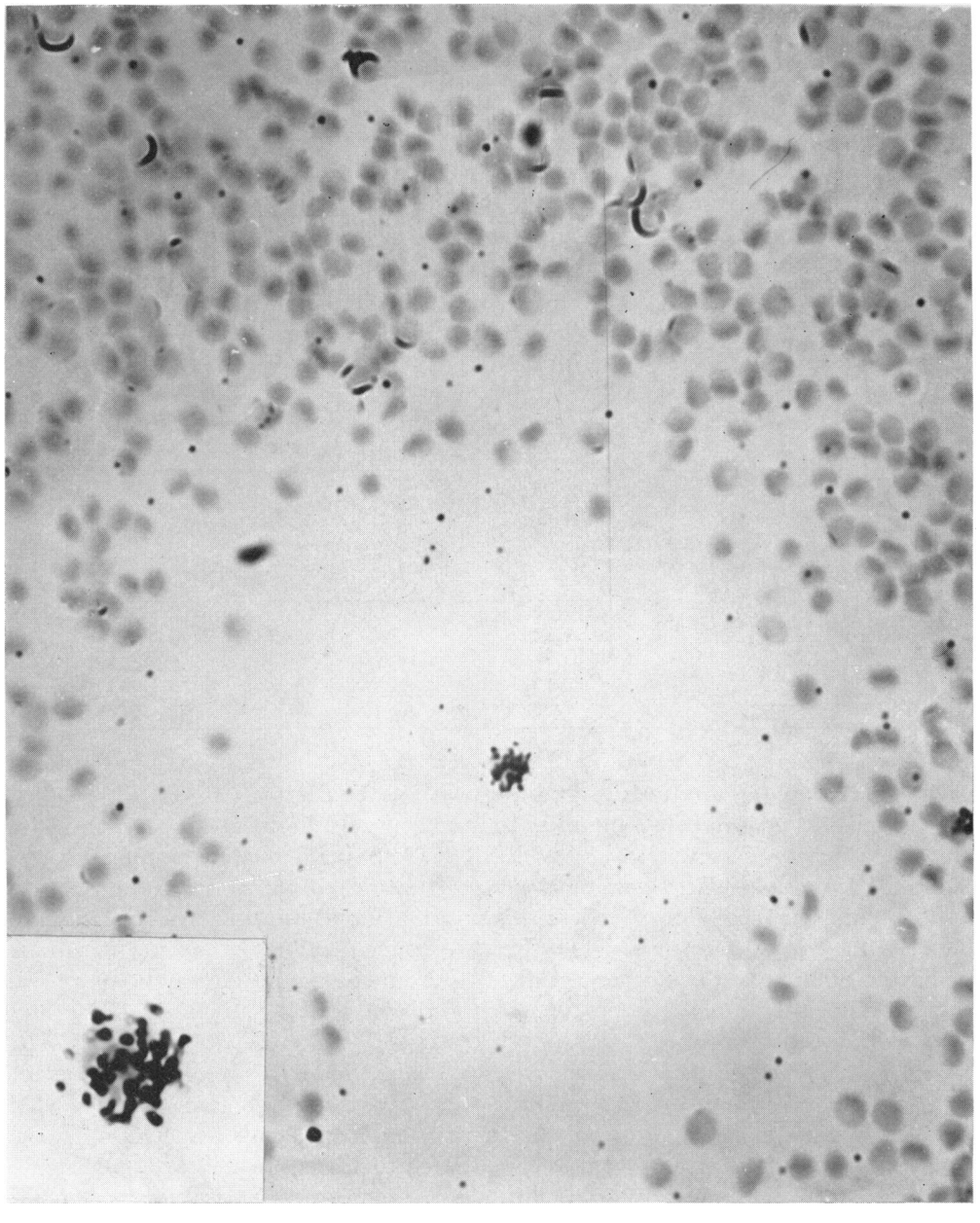

FIG. 1. Radioautograph of [^{3}H]thymidine labelled plaque-forming cell from mouse spleen fixed *in situ* on a monolayer of sheep erythrocytes.

Since the failure to rinse away all traces of glutaraldehyde leads to a high background grain count, thorough rinsing (30 minutes in running water) is recommended. 1 ml of emulsion spread in the petri dish and then drained off provided a good coating. For staining, any procedure which does not involve polystyrene solvents or high temperatures may be used. Giemsa is useful for routine work. Fig. 1 shows a radioautograph of the central cell of a plaque in a monolayer of sheep erythrocytes. A mouse in the exponential phase of its plaque-forming cell response to sheep erythrocytes was injected with [^{3}H]thymidine; 2 hours later its spleen cells were suspended and assayed. The preparation was fixed with glutaraldehyde vapour as described above, washed thoroughly, coated with Kodak Nuclear Track Emulsion NTB 3, and then developed and lightly stained with Giemsa 3 weeks later. The grains above the cell are obvious. Since the emulsion is in direct contact with the cell, there is no problem with loss of sensitivity caused by absorption of low-energy radiation.

DISCUSSION

The modified haemolytic plaque-forming cell assay described above makes feasible certain experimental approaches for which previous forms of the assay were ill-suited. Radioautography, morphological studies of either living or fixed plaque-forming cells, micro-manipulation of living plaque-forming cells, and the production of permanent preparations are all greatly facilitated by the absence of a gel support medium. Prior to fixation there is opportunity to wash off the cells being assayed without disruption of the bound erythrocyte indicator system; this permits accurate counts of plaques formed by small numbers of antibody-releasing cells present in suspensions whose concentration of inert cells would obscure any plaques produced in other assay systems. Finally, the sensitivity of the method is found comparable with the most sensitive of the previously available plaque-forming cell assays.

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