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SHEEP ERYTHROCYTE AND BLUETONGUE VIRUS ANTIBODY RESPONSES OF SPLEEN CELL CULTURES FROM MICE

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ABSTRACT

OELLERMANN, R. A. & CARTER, P., 1977. Sheep erythrocyte and bluetongue virus antibody responses of spleen cell cultures from mice. *Onderstepoort Journal of Veterinary Research*, 44 (3), 139–142 (1977).

The optimum conditions for the culture of cells from dissociated spleens were determined. Routinely, 10^7 cells were seeded per ml of RPMI 1640 medium supplemented with 20% pre-tested foetal calf serum. For the assay of the immune response, cultures were supplemented with 30 μMolar mercaptoethanol.

The immune responses to sheep erythrocyte and bluetongue virus antigens were determined by the haemolytic plaque-forming cell assays described by Oellermann (1974) and Oellermann, Carter & Marx (1976a). The optimum sheep erythrocyte antigen concentration was 2×10^6 erythrocytes per 10^7 spleen cells and maximum IgM plaque-forming cells were detected after 4 days in culture. Successful stimulation of the immune response to bluetongue virus was achieved in spleen cell cultures from mice previously primed with bluetongue virus. The optimum antigen concentration was 30–40 ng bluetongue virus per 10^7 spleen cells and the maximum plaque-forming cell response was observed after 4 days in culture.

Résumé

LA PRODUCTION D'ANTICORPS PAR DES CULTURES DE CELLULES SPLÉNIQUES DE SOURIS EN RÉPONSE AUX ÉRYTHROCYTES DE MOUTON ET AU VIRUS DE LA FIÈVRE CATARRHALE DU MOUTON

On a déterminé les conditions optimales pour la culture de cellules provenant de rates dissociées. La routine consistait à distribuer 10^7 *cellules par ml de milieu RPMI* 1640 *additionné de sérum foetal de veau à* 20%, *prétesté. Pour tester la réponse immunitaire, du mercaptoéthanol* 30 μ*molaire était ajouté aux cultures.*

La réponse immunitaire aux antigènes d'érythrocytes de mouton et du virus de la fièvre catarrhale du mouton ont été vérifiées au moyen du test d'hémolyse à cellules formatrices de plaques, tel que décrit par Oellermann (1974) *et Oellermann, Carter et Marx* (1976*a*). *La concentration optimale en antigènes d'érythrocytes de mouton a été déterminée à* 2×10^6 *érythrocytes par* 10^7 *cellules spléniques et les cellules formatrices de plaques à IgM ont été détectées au maximum après* 4 *jours en culture. On a réussi à exalter la réponse immunitaire au virus de la fièvre catarrhale du mouton en utilisant des cultures de cellules spléniques provenant de souris sensibilisées au préalable à ce même virus. La concentration optimale en antigène était de* 30–40 *ng de virus de fièvre catarrhale pour* 10^7 *cellules spléniques et la réponse maximale des cellules formatrices de plaques a été observée après* 4 *jours en culture.*

INTRODUCTION

Studies on the immunological response to bluetongue virus (BTV) *in vivo* in mice have recently been conducted (Oellermann, Carter & Marx, 1976b; Oellermann & Carter, 1977). However, to date, there have been no studies *in vitro* using BTV as antigen, but several reports have described the *in vitro* generation of primary and secondary immune responses by murine spleen cells to sheep erythrocyte (SRBC) antigen (Marbrook, 1967; Mishell & Dutton, 1967; Haskill, Byrt & Marbrook, 1970; Pierce, Johnson, Gershon & Asofsky, 1971). Considerable enhancement of antibody synthesis to SRBC *in vitro* was achieved by the addition of mercaptoethanol to the culture medium (Click, Benck & Alter, 1972 a).

The modified haemolytic plaque-forming cell (PFC) assay for the detection of BTV antibody-forming cells (Oellermann *et al.*, 1976a) results in small plaques. In the present study, *in vitro* SRBC PFC formation, being easier to demonstrate because of the size and clarity of the plaques, was studied first. This was followed by an analogous study of the BTV immune response, using spleen cells from mice previously primed with BTV.

MATERIALS AND METHODS

Antigens

The preparation of SRBC and BTV antigens has been described (Oellermann, 1974; Oellermann *et al.*, 1976a). Preliminary experiments showed that 2×10^6 SRBC or 30–40 ng BTV per 10^7 spleen cells per ml medium gave optimum results.

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Animals

Ten-week-old male albino mice from the colony maintained at the Institute were used. For each *in vitro* experiment, the spleens from a minimum of 3 mice were pooled.

Culture media

All the chemicals for the preparation of media were purchased from Merck Chemicals (Pty) Ltd, Johannesburg. Foetal calf serum (FCS), collected at the Pretoria abattoir, was filtered and tested for sterility and suitability for cell survival studies before use. Eagle's MEM and media F 12 and RPMI 1640 were prepared according to the formulations given in the Gibco Biocult catalogue, supplemented with varying concentrations of FCS, and tested. For routine purposes medium RPMI 1640 supplemented with 20% FCS was used.

After varying concentrations of spleen cell cultures for the routine assay of the immune response had been tested, they were supplemented with mercaptoethanol at a concentration of 30 μM.

Culture conditions

Triplicate cultures of varying concentrations of spleen cells were incubated in 60 mm plastic tissue culture dishes* containing 6 ml medium. Routinely, however, 10^7 spleen cells per ml medium were used. The stationary cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The assay for cell viability has been described (Oellermann, 1974).

* Lux obtained from Labretoria (Pty) Ltd, Pretoria

Assay of the immune response

Spleens from non-immunized mice were used to determine optimum culture conditions and to study the immune response to SRBC. However, the assay of the BTV PFC response in cultures of spleen cells from normal mice was unsatisfactory. To study its immune response, therefore, mice were primed individually with 40 μg BTV 10 days prior to the commencement of the *in vitro* experiments.

The haemolytic PFC assays for the determination of either SRBC or BTV specific antibody-forming cells have been described in detail (Oellermann, 1974; Oellermann *et al.,* 1976a). Only IgM PFC were assayed.

All the results presented are the averages of at least 3 experiments.

RESULTS

Survival of spleen cells seeded at different densities

To determine the optimum spleen cell concentration for cultures *in vitro,* $1-12\times10^7$ cells in 6 ml Eagle's MEM, supplemented with 15% FCS, were seeded per culture dish and their viability determined over a period of 6 days. The results are represented diagrammatically in Fig. 1.

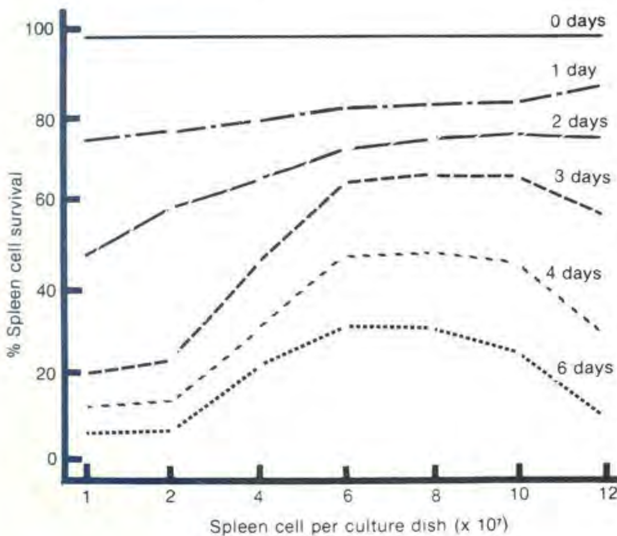

FIG. 1 The influence of the concentration of spleen cells on their survival after 0, 1, 2, 3, 4 and 6 days in culture

At low densities, spleen cell survival was extremely poor. The best cell survival was observed at $6-8\times10^7$ spleen cells per culture dish which is equivalent to $1-1,3\times10^7$ cells per ml. At higher concentrations a more rapid deterioration of cells was again observed. This was probably due to the depletion of essential nutrients from the medium. For routine purposes, therefore, cultures were seeded at 1×10^7 spleen cells per ml medium.

Survival of spleen cells in different media

Eagle's MEM and media F12 and RPMI 1640, supplemented with 12% FCS, were compared for their efficacy in maintaining spleen cells in culture. In addition the influence of RPMI 1640 supplemented with 6% and 20% FCS was investigated. The resulting cell survival curves over a period of 6 days in culture are delineated in Fig. 2.

The results show that medium RPMI 1640 was superior to both F 12 and Eagle's MEM. Furthermore, increasing the serum concentration from 6%–12% resulted in a marked improvement of the survival of spleen cells. Since the beneficial effect of an increase in the serum concentration to 20% was evident only during the longer periods of culture, RPMI 1640 supplemented with 20% FCS was used in all the ensuing work.

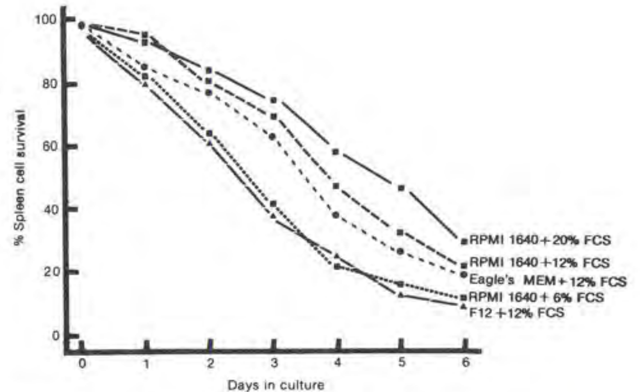

FIG. 2 The influence of different culture media on spleen cell survival *in vitro:* RPMI 1 640+20% FCS; RPMI 1 640+12% FCS; RPMI 1 640+6% FCS; Eagle's MEM+12% FCS; F12+12% FCS

In vitro *immune response of spleen cell cultures to SRBC*

Spleen cell cultures were supplemented with 3–300 μM mercaptoethanol and stimulated with 10^7 SRBC per dish. The PFC response was determined after 4 days in culture. The results are represented in Fig. 3.

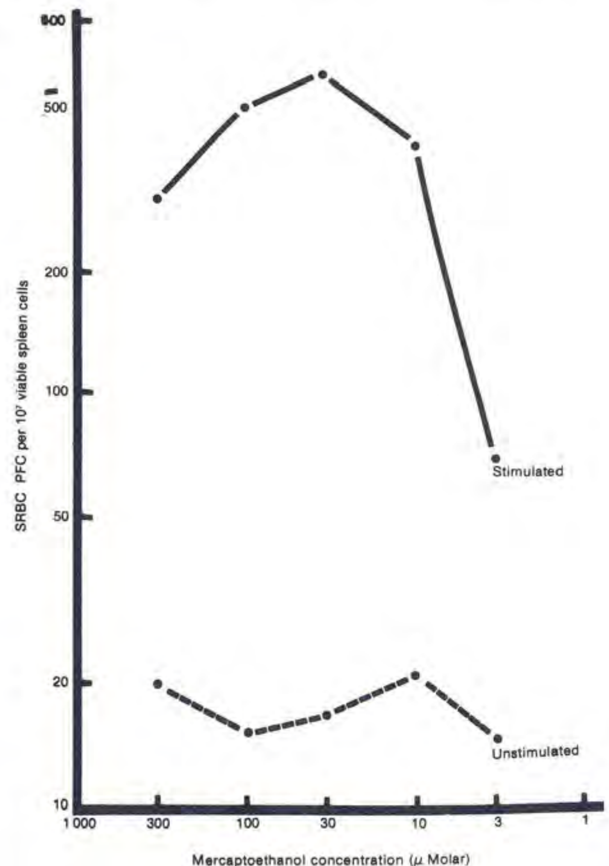

FIG. 3 The influence of varying concentrations of mercaptoethanol supplementation on the SRBC PFC response of spleen cell cultures stimulated and unstimulated with SRBC

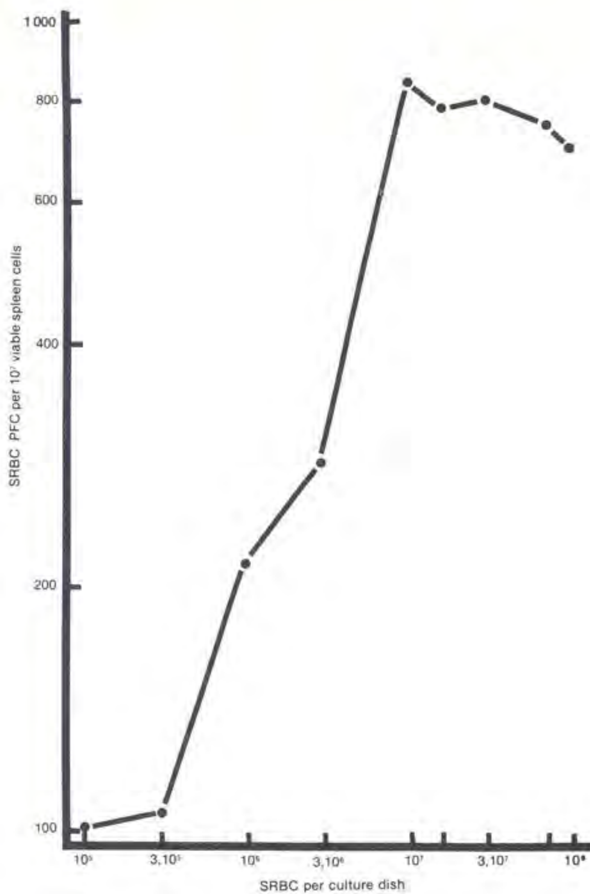

FIG. 4 The SRBC PFC response of spleen cell cultures stimulated with varying concentrations of SRBC antigen

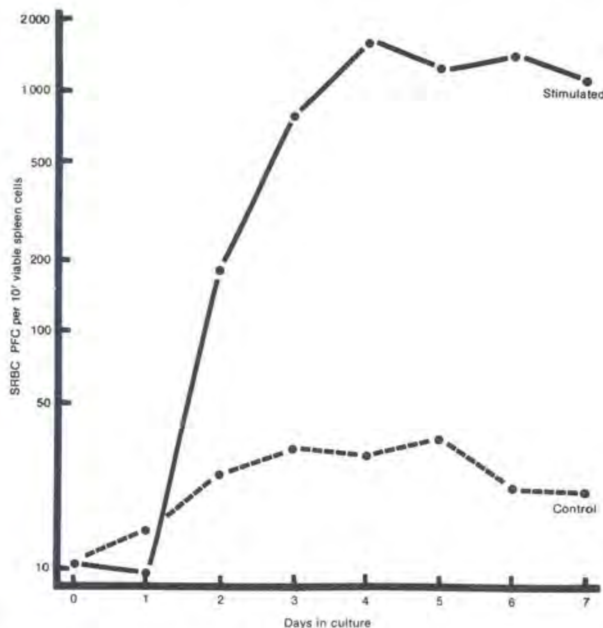

FIG. 5 The SRBC PFC time response of SRBC stimulated and control spleen cell cultures

The optimum concentration was found to be 30 μM mercaptoethanol which was similar to the optimum of 20 μM determined by Click *et al.* (1972a). Mercaptoethanol supplementation of spleen cell cultures was therefore routinely used.

In the following experiments the influence of 10^5–10^8 SRBC antigen on the PFC response of spleen cells after 4 days in culture was determined. The results are presented in Fig. 4.

The optimum antigen concentration was found to be approximately $1,2\times10^7$ SRBC per dish, representing 2×10^6 SRBC per 10^7 spleen cells.

The appearance of SRBC PFC after increasing periods in culture was studied next. The results are represented in Fig. 5.

A relatively low background of spontaneous SRBC PFC was found in control cultures. Stimulation with SRBC resulted in a marked increase in PFC above that of the control after 2 days in culture, reaching a maximum after 4 days. Thereafter, practically no decrease occurred up to Day 7, an indication that the stimulated cells remained active for some time *in vitro*. These results are different from the response *in vivo* (Oellermann *et al.*, 1976 b) in which the IgM PFC reached a peak after 4 days, and declined rapidly thereafter until Day 8.

In vitro *immune responses to BTV*

Spleen cell cultures from non-immunized mice were stimulated with varying concentrations of BTV and assayed for their PFC response. When the slides were examined microscopically small areas of lysis surrounding central lymphocytes could be detected but the assay was considered to be unsatisfactory. Mice were therefore primed with 40 μg BTV and spleen cell cultures prepared 10 days later. These cultures were then again stimulated with varying concentrations of BTV and the PFC response assayed after 3–4 days. The results are presented in Table 1.

TABLE 1 The *in vitro* stimulation of sensitized mouse spleen cell cultures by different concentrations of BTV

Mice primed with BTV	ng BTV per 10^7 spleen cells	PFC per 10^7 viable spleen cells
+	333	475
+	67	680
+	33	940
+	3	500
+	0	160
−	0	27

A BTV antigen concentration-dependent stimulation of spleen cells from primed mice was clearly demonstrated. Optimum stimulation was obtained with approximately 200 ng BTV per culture dish which is equivalent to 30–40 ng BTV per 10^7 spleen cells.

The BTV PFC time response of cultures stimulated with 30–40 ng BTV per 10^7 spleen cells was determined and the results are shown in Fig. 6.

The maximum stimulation of BTV PFC *in vitro* above the values found in primed cultures was observed after 4 days. This response curve was similar to that of the secondary IgM PFC response to BTV *in vivo* (Oellermann *et al.*, 1976b).

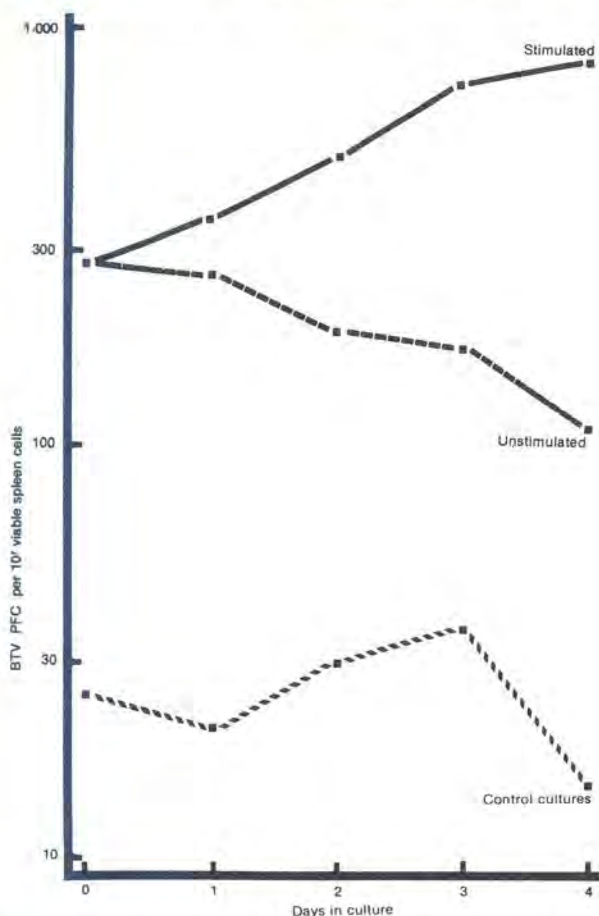

FIG. 6 The BTV PFC time response of BTV stimulated and unstimulated BTV mouse-primed spleen cell cultures and control cultures from non-primed mice

Discussion

During the collection of FCS, blood was taken from individual foetuses and the resultant sera were tested for their ability to maintain spleen cell cultures. Frequently, batches of FCS were found to be inferior and unsuitable for *in vitro* studies. Although an explanation for this was not sought, the phenomenon appears to be a general one, as other workers clearly specify the serum batches used (Pierce *et al.*, 1971).

In the present investigation, additional nutritional supplementation of RPMI 1640 during the 6-day-period of culture did not improve the survival of spleen cells (Carter, unpublished observation). This differs from the reports by Pierce *et al.*, (1971) and Click *et al.* (1972b). Both groups of workers, however, used Eagle's MEM as basic medium. This could explain the difference in results, as RPMI 1640 was shown to be superior to Eagle's MEM for the maintenance of spleen cells in culture.

The experimental results shown in Fig. 1 demonstrate that in low concentrations, spleen cells did not survive for any extended periods in culture. At higher concentrations of $1\text{–}1,3 \times 10^7$ cells per ml, however, a marked improvement in the cell survival was observed. This could be explained by the conditioning effect on media by both primary (Rubin, 1966) and line cells (Oellermann & Miller, 1969) in culture. Conditioned medium, obtained from high density cultures, had a marked growth stimulatory effect on cells seeded at low concentrations. In primary cell cultures the conditioning effect was attributed to a heat labile macromolecule whereas in line cell cultures essentially dialyzable factors seemed to be responsible.

The *in vitro* response to SRBC was of the order of $1,6 \times 10^3$ PFC per 10^7 viable spleen cells which was considerably less than that reported by Click *et al.*, (1972b) for spleen cell cultures from C_{57}Bl mice. The *in vitro* response was, however, only approximately 3 times less than the PFC response determined *in vivo* (Oellermann *et al.*, 1976b). This emphasizes the fact that genetic differences between strains of mice are responsible for the differences in their immune responses.

The induction of the immune response to BTV in spleen cells from non-immunized mice appeared to be ineffectual. Since successful stimulation of spleen cells in culture by BTV could only be obtained with cells from previously immunized mice, it appears that spleen cell cultures from unprimed mice have insufficient cells capable of processing the antigen. Mice were primed by the intraperitoneal injection of BTV and, as macrophages are present in large numbers in the peritoneal cavity, their role in the processing of BTV and assistance in the successful induction of immunocompetent lymphocytes is indicated. It should be possible to resolve this problem by further studies *in vitro* of cellular interactions in the immune response to BTV.

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